

Complete suspension culture of human induced pluripotent stem cells supplemented with suppressors of spontaneous differentiation

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Abstract

Human induced pluripotent stem cells (hiPSCs) are promising resources for producing various type of tissues in regenerative medicine; however, a scalable culture system that can precisely control the cell status for hiPSCs is not developed yet. Utilizing suspension culture without microcarriers or special materials allows for massive production, automation, cost effectiveness, and safety assurance in industrialized regenerative medicine. Here, we found that hiPSCs cultured in suspension conditions with continuous agitation without any microcarriers or extracellular matrix components were more prone to spontaneous differentiation than those cultured in conventional adherent conditions. Adding PKC β and Wnt signaling pathway inhibitors in the suspension conditions suppressed the spontaneous differentiation of hiPSCs into ectoderm and mesendoderm, respectively. In these conditions, we successfully completed culture processes of hiPSCs including the generation of hiPSCs from peripheral blood mononuclear cells with the expansion of bulk population and single-cell sorted clones, long-term culture with robust self-renewal characteristics, single cell cloning, direct cryopreservation from suspension culture and their successful recovery, and efficient mass production of a clinical-grade hiPSC line. Our results demonstrate that precise control of hiPSC status in suspension culture conditions paves the way for their stable and automated clinical application.

eLife assessment

The authors have identified a media condition that maintains iPSCs in suspension cultures by inhibiting the PKC β and Wnt signaling pathways. The manuscript is **valuable** for the pluripotent stem cell field. This is a **solid** study with substantial data to support the conclusions in the manuscript.

Introduction

Human induced pluripotent stem cells (hiPSCs) are promising resources for various types of tissues in regenerative medicine (Takahashi *et al*, 2007 [↗](#); Yu *et al*, 2007 [↗](#)). To enable cell therapy from hiPSCs, the development of a large-scale manufacturing system is essential because massive cell numbers are required to compose transplantable cells which are enough to rescue the desired physiological function (Chen *et al*, 2014 [↗](#); Kim & Kino-Oka, 2020 [↗](#); Tannenbaum & Reubinoff, 2022 [↗](#)). In general, hiPSCs are believed to possess their scaffold dependency, thus are cultured under adhesion and monolayer culture conditions (Hayashi & Furue, 2016 [↗](#); Xu *et al*, 2001 [↗](#)). However, utilizing suspension culture without microcarriers or special materials allows for massive production, automation, cost-effectiveness, and safety assurance in industrialized regenerative medicine.

Several attempts have been made to develop suspension culture technologies enabling rapid and large-scale preparation of hiPSCs (Amit *et al*, 2010 [↗](#); Amit *et al*, 2011 [↗](#); Dang *et al*, 2004 [↗](#); Elanzew *et al*, 2015 [↗](#); Horiguchi & Sakai, 2016 [↗](#); Hunt *et al*, 2014 [↗](#); Ibuki *et al*, 2019 [↗](#); Kehoe *et al*, 2010 [↗](#); Kim *et al*, 2019 [↗](#); Krawetz *et al*, 2010 [↗](#); Kwok *et al*, 2018 [↗](#); Lam *et al*, 2016 [↗](#); Nath *et al*, 2017 [↗](#); Oh *et al*, 2009 [↗](#); Olmer *et al*, 2012 [↗](#); Rohani *et al*, 2020 [↗](#); Shafa *et al*, 2012 [↗](#); Singh *et al*, 2010 [↗](#); Steiner *et al*, 2010 [↗](#); Wang *et al*, 2013 [↗](#); Zweigerdt *et al*, 2011 [↗](#)) (Summarized in **Table 1** [↗](#)). These studies have achieved long-term culture and/or mass expansion of hiPSCs and/or human embryonic stem cells (hESCs) in suspension conditions. However, completed processes from clonal hiPSC generation to mass production of hiPSCs based on the precise control of cell status have not yet been achieved.

In this study, we have investigated what hampers the stable maintenance of undifferentiated cell states in suspension conditions. HiPSCs cultured in suspension conditions with continuous agitation without any microcarriers or extracellular matrix (ECM) components were more prone to spontaneous differentiation than those cultured in conventional adherent conditions. From screening of candidate molecules to suppress the spontaneous differentiation of hiPSCs, we have identified that inhibitors of PKC β and Wnt signaling pathways suppress their differentiation into ectoderm and mesendoderm, respectively. In these conditions, we aimed to complete the processes of handling hiPSCs including the generation of hiPSCs with the expansion of bulk population and single-cell sorted clones, long-term culture with robust self-renewal characteristics, single-cell cloning, direct cryopreservation from suspension culture and their successful recovery, and efficient mass production of a clinical-grade hiPSC line.

Results

Suspension cultured hiPSCs are prone to spontaneous differentiation

First, we investigated whether the quality of hiPSCs in suspension and adherent conditions are equivalent or not. HiPSCs cultured in a conventional medium with continuous agitation (90 rpm) in non-adhesive cell culture plates for 2 passages (5 days during passages) were examined (**Figure 1A** [↗](#)). In suspension culture on day 5 and 10, hiPSCs formed round cell assemblies with slightly uneven surfaces (**Figure 1B** [↗](#)). Gene expression analysis with RT-qPCR revealed that the expression of differentiation markers, such as PAX6 (ectoderm), SOX17 (endoderm), and T (mesoderm) increased in suspension-cultured hiPSCs for 10 days (**Figure 1C** [↗](#)). To monitor the spontaneous differentiation at single cell resolution, we established knock-in reporter hiPSC lines of PAX6-tdTomato and SOX17-tdTomato to visualize and quantify the expression of PAX6 and

Article	Medium	Additives	Cell lines tested	Scalability	Transcriptome	Single cell cloning	Direct freeze and thaw	iPSC generation
Matsuo-Takasaki et al. 2023 (This study)	AK02N, AK03N, StemScale	Y-27632, IWR-1-endo, LY333531	hiPSC (WTc11, 201B7, 1383D6, 1231A3, Ff-114s04)	$\sim 10^9$	Yes	Yes	Yes	Yes
Amit et al., 2010, 2011	DMEM/F12 + KSR	Y-27632, LIF, bFGF	hESC (I3, Ie, I5, H9.2, H9, H7, H14) hiPSC (iF4, J1.2.3, C3, C2, KTN7, KTN13)	$\sim 10^7$	No	No	No	No
Krewetz et al., 2010	mTeSR1	Y-27632, Rapamycin	hESC (H9)	$\sim 10^8$	No	No	No	No
Singh et al., 2010 Zweigerdt et al., 2011 Olmer et al., 2012	KnockOut DMEM + KSR, mTeSR1	Y-27632, bFGF	hESC (hES2, hES3, and ESI049) hiPSC (hCBiPS2, hiPSOCT4eGFP)	$\sim 10^8$	No	No	No	No
Steiner et al., 2010	Neurobasal medium + KSR	Y-27632, bFGF, Activin A, Fibronectin, Gelatin, BDNF, NT3, NT4, Nutridoma-CS	hESC (HES1, HES2, H7)	$\sim 10^6$	No	No	No	No (Of note, 3 hESC lines derivation)
Wang et al., 2013	TeSR-E8	Y-27632	hiPSC (BC1, TNC1)	$\sim 10^8$	No	No	No	No
Hunt et al., 2014	mTeSR1	Y-27632	hESC (H1, H9)	$\sim 10^7$	No	No	No	No
Elanzew et al., 2015	mTeSR1, TeSR-E8	Y-27632	hESC (H9) and hiPSC (iLB-C-31f-r1)	$\sim 10^7$	No	No	No	No
Horiguchi et al., 2016; Ibuki et al., 2019	TeSR-E8, mTeSR1	Y-27632, KSR, Albumax, LPA, SIP,	hiPSC (TkDN4-M, TkDA3-4, 201B7)	$\sim 10^6$	No	No	No	No
Nath et al., 2017	Dialyzed DMEM/F-12	Y-27632, bFGF, TGF- β 1	hiPSC (Tic)	$\sim 10^8$	No	No	No	No
Kwok et al, 2018	mTeSR1, StemMACS	Y-27632	hiPSC (AR1034ZIMA) hiPSC clone1, FS hiPSC clone2)	$\sim 10^9$	No	No	No	No
Rohani et al., 2020	mTeSR1, RSeT	Y-27632, Rapamycin	hESC (H1, H9)	$\sim 10^7$	Yes	No	No	No

Table 1.

The list of published study on scalable suspension culture without microcarriers for hPSCs.

SOX17 at protein level, respectively (**Figure 1—figure supplement 1A–H**). tdTomato-positive cells were clearly observed in day 10 samples in suspension-cultured hiPSCs, whereas no positive cells were observed in adherent culture conditions (**Figure. 1D**). Flow cytometric analysis revealed that hiPSCs in suspension-culture conditions contained non-negligible percentages of PAX6-tdTomato-positive and SOX17-tdTomato-positive cells (**Figure. 1E** and **1F**). Western blot analysis revealed that protein expression of PAX6 and SOX17 in suspension-culture was significantly increased (**Figure 2—figure supplement 1A–D**). The ratio of positive cells for a cell surface marker for undifferentiated hiPSCs, TRA1-60, was significantly lower in suspension-culture conditions (**Figure 2—figure supplement 1E** and **F**). These results suggest that a portion of hiPSCs was spontaneously differentiated in suspension conditions when cultured in conventional medium. To examine global changes in gene expression patterns between suspension and adherent conditions, whole-transcriptomic RNA-seq experiments with statistical tests were performed. Gene Set Enrichment Analysis (GSEA) of all the genes and Gene Ontology Enrichment Analysis (GOEA) on differentially-regulated genes revealed that, in suspension conditions, number of genes involved in differentiation toward various tissues and cell-cell adhesions were significantly up-regulated, whereas genes involved in nucleotide metabolism, hypoxic responses, and extracellular matrix organization were significantly-down-regulated (**Figure 1G–J**). These results suggest that hiPSCs in suspension conditions are in the process of spontaneous differentiation into various cell lineages and are characterized with specific signatures of gene expression patterns.

Wnt signaling inhibitors suppress spontaneous mesendodermal differentiation in suspension culture condition

We next investigated the factors having an inhibitory effect on hiPSCs spontaneous differentiation in suspension conditions (**Figure 2A**). Since Wnt signaling induces the differentiation of human pluripotent stem cells into mesendoderm lineages (Nakanishi *et al*, 2009; Sumi *et al*, 2008; Tran *et al*, 2009; Vijayaragavan *et al*, 2009; Woll *et al*, 2008), and is endogenously involved in the regulation of mesendoderm differentiation of pluripotent stem cells (Dziedzicka *et al*, 2021), we hypothesized that adding Wnt signaling inhibitors/activator may alter spontaneous differentiation of hiPSCs into mesendoderm. Therefore, Wnt signaling inhibitors, IWP2 or IWR-1-endo (Chen *et al*, 2009), or an activator, CHIR99021 (Ring *et al*, 2003), were added to the culture medium under suspension conditions. HiPSC aggregates treated with and without inhibitors showed similar shapes (**Figure 2B**). In contrast, hiPSC aggregates treated with CHIR99021 formed heterogeneously shaped cyst-like structures, suggesting that they were largely differentiated. In samples treated with inhibitors, both T and SOX17 expression levels were significantly reduced to the level of adherent-cultured hiPSCs; however, there was no reduction in PAX6 expression (**Figure 2C**). In contrast, CHIR99021-treated cell aggregates showed markedly decreased OCT4 and increased T and SOX17 expression. Additionally, SOX17 protein expression was suppressed in hiPSCs treated with IWR-1-endo in suspension-culture conditions, although its expression increased in hiPSCs in suspension conditions with conventional culture medium compared to adherent conditions (**Figure 2—figure supplement 1A** and **B**). These results indicate that Wnt signaling inhibitors effectively suppressed mesendodermal differentiation in suspension-culture, but was insufficient to suppress ectodermal differentiation.

PKC signal inhibitors suppress spontaneous neuroectodermal differentiation in suspension conditions

To identify molecules with inhibitory activity on neuroectodermal differentiation, hiPSCs were treated with candidate molecules in suspension conditions. Out of the candidate molecules tested (listed in **Supplementary Table 1**), Go6983, a pan-PKC inhibitor (Gschwendt *et al*, 1996), showed the strongest inhibition against PAX6 expression (**Figure 2D**). Further, simultaneous treatment with Go6983 and IWR-1-endo decreased PAX6, T, and SOX17 expression, while

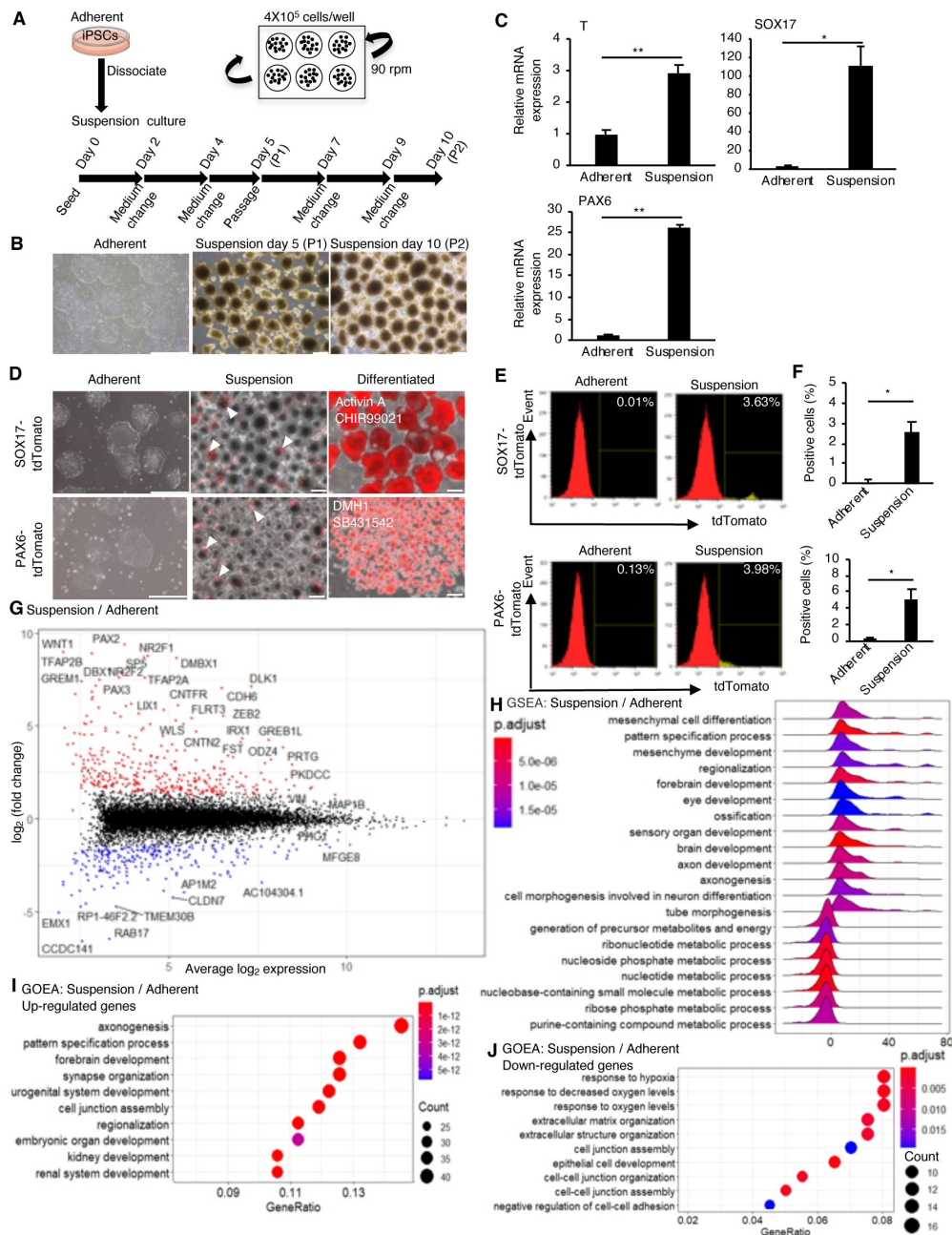


Figure 1.

HiPSCs maintained under suspension-culture conditions undergo spontaneous differentiation.

(A) Schematics representing hiPSCs in suspension-culture conditions. (B) Phase-contrast images of adherent- or suspension-cultured hiPSCs on day 5 (Passage 1 (P1)) and day 10 (Passage 2 (P2)). Scale bars: 400 μ m. (C) Gene expression in hiPSCs cultured under adherent or suspension conditions on P2. Bar graphs show the mean $\pm$ SE (n=3). P-values were statistically analyzed by Student's t-test. (D) Phase-contrast and fluorescent images of adherent or suspension-cultured reporter hiPSCs on P2. White arrowheads indicate spontaneous expression of SOX17 and PAX6 in suspension conditions. Scale bars: 400 μ m. (E) Quantification of hiPSCs spontaneous differentiation with flow cytometry. (F) Averaged tdTomato-positive cell ratio (%) from flow cytometry data (mean $\pm$ SE from n=3). P-values were statistically analyzed by Student's t-test. (G) An MA plot comparing transcriptomes between suspension- and adherent-culture from RNA-seq data. The representative gene name is shown in the plot. (H) GSEA on the gene sets of suspension-cultured hiPSCs to adherent cultures. Adjusted P-values are shown as blue to red from low to high values. (I and J) GOEA on the gene sets of suspension-cultured hiPSCs to adherent culture. Results are ranked by significance (p-adjusted value) and/or counted gene numbers.

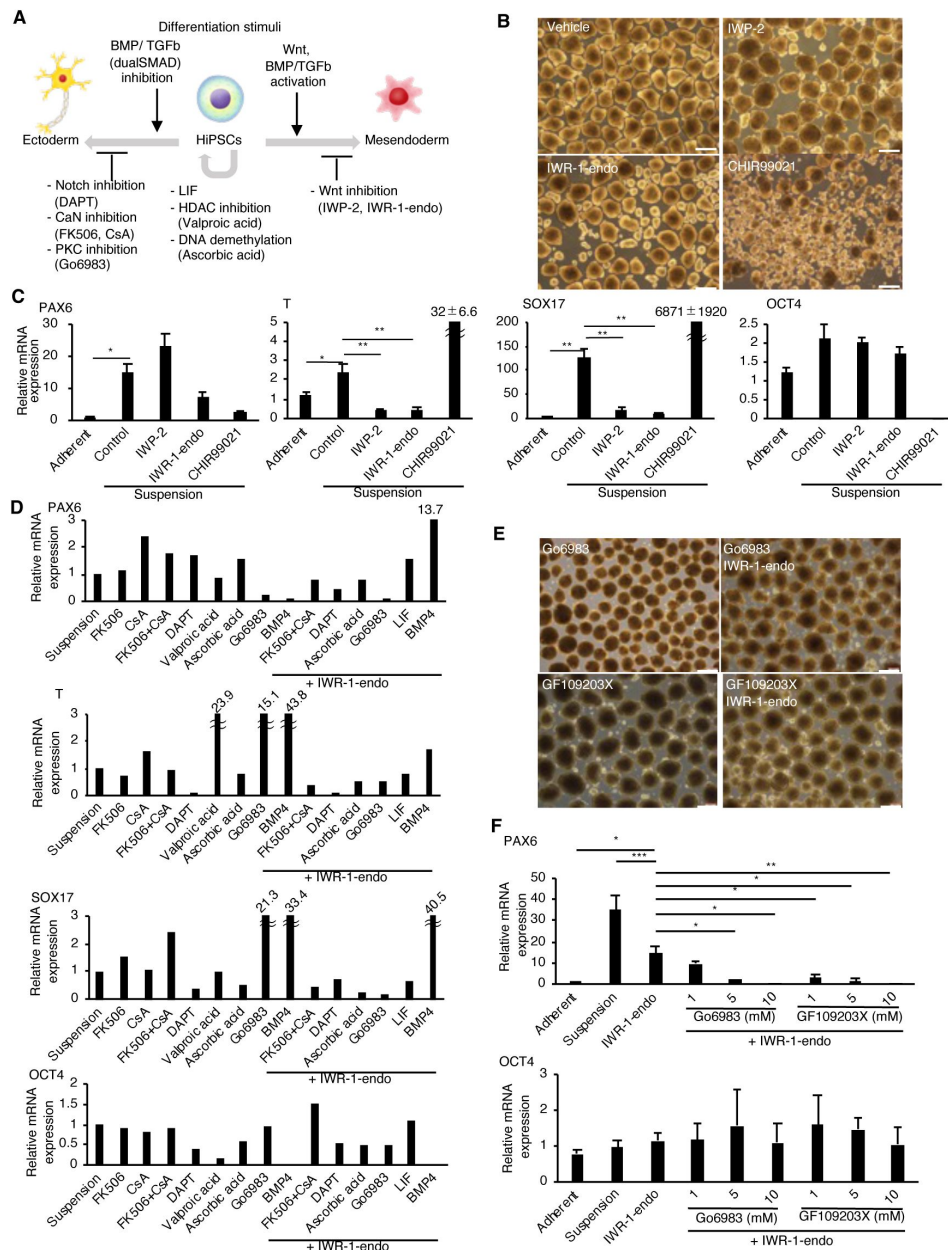


Figure 2.

PKC inhibitors suppress spontaneous differentiation of hiPSCs into neural ectoderm in suspension-culture conditions.

(A) Schematics. (B) Phase-contrast pictures of suspension-cultured hiPSCs in the presence of Wnt signaling inhibitors (IWP-2 and IWR-1-endo) or activator (CHIR99021). Scale bars: 400 μ m. (C) Gene expression of hiPSCs in suspension-culture conditions with or without IWP2, IWR-1-endo or CHIR99021. RT-qPCR was performed on day 10 samples (P2). Gene expressions were normalized to GAPDH and displayed as relative fold increase to adherent-cultured samples. Bar graphs show the mean $\pm$ SE. P-values were statistically analyzed using Dunnett's multiple comparisons test. (D) Screening of inhibitory activity of candidate molecules on neuroectoderm differentiation in suspension-cultured hiPSCs. Candidate molecules were added in combination as shown. Results are displayed as relative fold increase to suspension-cultured samples without pharmacological treatment. n=1. (E) Phase-contrast images of suspension-cultured hiPSCs on P2 in the presence of PKC inhibitors (Go6983 or GF109203X) alone, or in combination with IWR-1-endo. Scale bars: 400 μ m. (F) Suspension-cultured hiPSCs gene expression in the presence of IWR-1-endo or with combined IWR-1-endo and different doses of PKC inhibitors. Results are displayed as relative fold increase to adherent-culture. Data are presented as mean $\pm$ SE (n=3).

maintaining OCT4 expression. To confirm these screening test results, the dose dependent effect of Go6983 or another PKC α , β , γ inhibitor GF109203X (GFX) (Toullec *et al.*, 1991) on the inhibition of PAX6 expression was observed at constant concentration of IWR-1-endo (**Figure 2E** and **F**). These results demonstrated that the inhibition of PKC signaling pathway effectively suppress neuroectoderm differentiation in hiPSCs cultured in suspension conditions. Furthermore, we tested various PKC inhibitors to suppress neuroectodermal differentiation from hiPSCs in suspension culture using the 201B7 hiPSC line (**Figure 2—figure supplement 2A**). The suppression of PAX6 expression in hiPSCs cultured in suspension conditions was observed with PKC inhibitors, enzastaurin (Ly317615), sotrastaurin (AEB071), Ro-32-0432, Go6983, GF109203X, and LY333531, which possessed PKC β inhibition activity (**Figure 2—figure supplement 2B-D**); however, sotrastaurin and Ro-32-0432 also showed growth inhibition. Thus, for the rest of the study, a PKC β specific inhibitor, LY333531 (Jirousek *et al.*, 1996) was used. As compared to controls, another hiPSC line, WTc11, cultured in suspension conditions treated with IWR-1-endo and LY333531 formed homogenous, round, smooth-surfaced aggregates (**Figure. 3A**). PAX6 expression was strongly suppressed by the addition of LY333531 (**Figure. 3B**). Furthermore, after adding IWR-1-endo, the inhibitory effect of PAX6 expression was further enhanced, and simultaneously, OCT4 expression was restored to the same level as in the adherent-culture. PAX6 protein expression was also suppressed in hiPSCs treated with IWR-1-endo and LY333531 in suspension-culture conditions while its expression increased in suspension conditions with conventional culture medium compared to adherent-conditions (**Figure 2—figure supplement 1C** and **D**). The ratio of TRA-1-60-positive cells was higher in suspension-culture conditions supplemented with IWR-1-endo and LY333531 than in control conditions without these inhibitors (**Figure 2—figure supplement 1E** and **F**). These results indicate that the maintenance of suspension-cultured hiPSCs is specifically facilitated by the combination of PKC β and Wnt signaling inhibition.

We examined the effect of PKC β and Wnt inhibitors on global gene expression of hiPSCs under suspension conditions. RNA-seq data were obtained from suspension conditions in absence of inhibitors (Sus), supplemented with IWR-1-endo (IWR), LY333531 (LY), IWR-1-endo and LY333531 (IWRLY), and adherent conditions (Ad). Hierarchical clustering obtained from these data showed that LY and IWRLY were grouped closely with Ad (**Figure 3C**). In contrast, Sus and IWR were both grouped as discrete populations from Ad. Additionally, hierarchical clustering in gene expression among these conditions was supported by principal component 1 (PC1) in the principal component analysis (PCA) (**Figure 3D**). In contrast, PC2 represented genes related to the effects of specific inhibitors under these conditions. Next, we investigated the effect of LY333531 and IWR-1-endo in suspension culture. Many genes involved in pluripotency: KLF4 and ID1, and epithelial cell-cell interactions: CDH1 (E-cadherin), were significantly up-regulated in IWRLY, while many transcription factors involved in differentiation: PAX2, 3, 5, and 8, SP5, DBX1, and TFAP2B, were down-regulated in IWRLY (**Figure 3E**). GSEA and GOEA on down-regulated genes in IWRLY showed that the expression of developmentally associated genes, whose expression was elevated in Sus, was generally reduced in IWRLY (**Figure 3F** and **G**). GOEA on up-regulated genes revealed gene sets involved in epithelial cell types (**Figure 3H**). As compared to Ad, genes involved in sensory system development, cell-cell adhesion, and Wnt and PI3K signaling pathways were up-regulated in IWRLY, and genes involved in nucleotide metabolism and hypoxic responses were down-regulated under IWRLY conditions (**Figure 3—figure supplement 1A-D**). These results suggest that PKC β and Wnt signaling inhibitors in suspension conditions regulate global gene expression patterns to suppress spontaneous differentiation, albeit remaining expression signatures of suspension culture, possibly due to the microenvironment within the formed aggregates and physiological differences.

Further, we examined the expression pattern changes in specific isoforms of PKCs in hiPSCs cultured in adherent/suspension conditions. RNA expression of PKC α (PRKCA) and PKC β (PRKCB) was significantly up-regulated under suspension-culture conditions compared to adhesion conditions (**Figure 3—figure supplement 2A**). Moreover, phospho-PKC β protein expression was

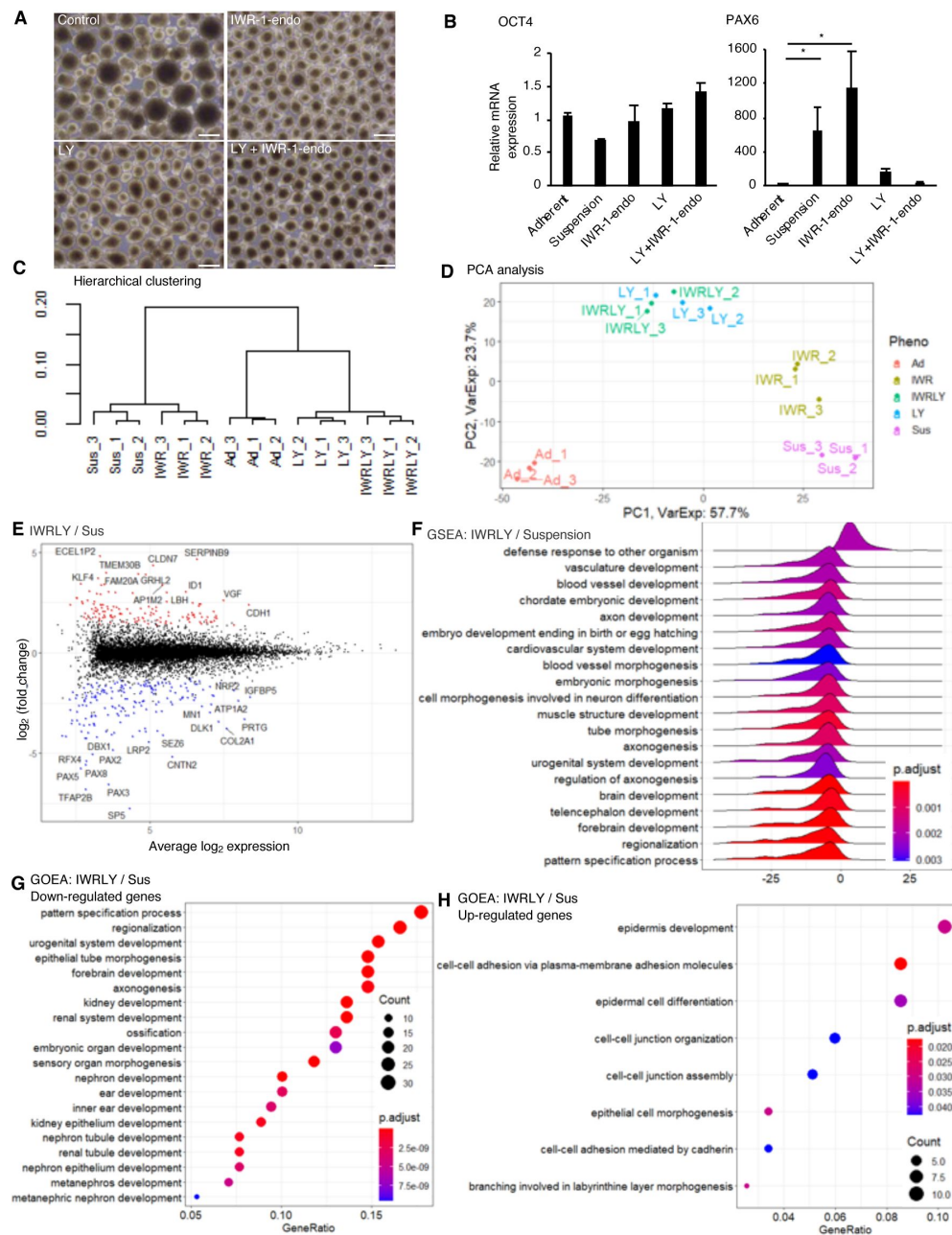


Figure 3.

The inhibitors of WNT and PKC β efficiently maintain the self-renewal of hiPSCs in suspension conditions.

(A) Phase-contrast images of suspension-cultured hiPSCs on P2 in the presence of IWR-1-endo, LY333531, or both. Scale bars: 400 μ m. (B) Gene expression of suspension-cultured hiPSCs on P2 in the presence of IWR-1-endo, LY333531, or both. Data are presented as mean $\pm$ SE. P-values were statistically analyzed with Dunnett's test. (C) Hierarchical clustering of adherent and suspension-cultured hiPSCs on P2 using Ward's method from RNA-seq data (n=3 in each condition). Ad, Adherent; Sus, Suspension; IWR, IWR-1-endo; LY, LY333531; IWRLY, IWR-1-endo and LY333531. (D) Principal component analysis (PCA) plot showing clusters of samples based on similarity. Gene expression variance are displayed as PC1 and PC2. (E) MA plot comparing transcriptomes between IWRLY and Sus. (F) GSEA on the gene sets of IWRLY to Sus from these RNA-seq data. Statistically significant enrichment is shown. P-values are represented in blue to red from low to high values. (G and H) GOEA for the gene sets of IWRLY to Sus from these RNA-seq data. Analysis was performed on down-regulated genes in (G) and up-regulated genes in (H).

significantly elevated (**Figure 3—figure supplement 2B** [↗](#) and **C** [↗](#)). These results suggest that elevated expression of PKC β in suspension-cultured hiPSCs could affect the spontaneous differentiation.

Simultaneous inhibition of PKC β and Wnt signals maintains undifferentiated hiPSCs in suspension for long-term culture

We next performed long-term culture for 10 passages in suspension conditions and compared hiPSC growth in presence of LY333531 or Go6983. When hiPSCs were seeded at 4×10^5 cells/well, the average cell number reached approximately 12-fold after 5 days under both conditions (**Figure 4A** [↗](#) and **B** [↗](#)). After 10 passages, aggregates of hiPSCs cultured in the presence of LY333531 showed a uniform spherical shape, whereas aggregates cultured in the presence of Go6983 were heterogeneously spherical (**Figure 4C** [↗](#)). Notably, in LY333531-treated cells, OCT4-positive cell numbers were significantly higher than in Go6983-treated samples, as determined by immunostaining (**Figure 4D** [↗](#) and **E** [↗](#)). To evaluate whether hiPSCs cultured in suspension conditions with PKC β and Wnt signaling inhibitors for 10 passages maintain pluripotency, we performed embryoid body (EB) formation assay. These EBs contained positive cells for TUJ1, SMA, and AFP as ectodermal, mesodermal, and endodermal marker, respectively (**Figure 4F** [↗](#)). Comparative genomic hybridization (CGH) array analysis showed that hiPSCs cultured long-term in the presence of PKC β and Wnt inhibitors retained their normal human karyotype (**Figure 4G** [↗](#)). These results indicate that, for long-term culture, inhibition of Wnt signaling and PKC β in suspension conditions was sufficient to maintain the self-renewal, pluripotency, and genomic integrity of hiPSCs.

We further investigated whether the effects of PKC β and Wnt inhibitors on suppressing hiPSCs spontaneous differentiation in suspension-culture conditions are applicable to other culture medium. Morphologies and gene expression profile of hiPSCs, cultured in suspension conditions with another commercially available maintenance medium, StemScale (ThermoFisher Scientific, Inc., MA, USA), were examined (**Figure 4—figure supplement 1A** [↗](#)). HiPSCs cultured in suspension treated with IWR-1-endo and LY333531 formed homogeneously round and smooth-surfaced aggregates compared to controls (**Figure 4—figure supplement 1B** [↗](#)). The differentiation markers expression, which was elevated under suspension conditions, was suppressed by the simultaneous addition of PKC β and Wnt signal inhibitors, as observed with StemFit medium (**Figure 4—figure supplement 1C** [↗](#) and **D** [↗](#)). These results suggest that conventional suspension culture methods might contain spontaneous differentiating cells and that the addition of inhibitors of PKC β and Wnt signaling pathways to culture medium generally suppresses spontaneous differentiation.

Mass and clonal expansion of hiPSCs in suspension conditions supplemented with inhibitors of PKC β and Wnt signaling pathways

For the clinical applications of hiPSCs, its homogenous mass production is required to obtain sufficient quantities. To test the feasibility of mass production under suspension-culture conditions supplemented with PKC β and Wnt signal inhibitors, we first performed suspension-culture using healthy-donor derived hiPSC lines, 1383D6 and 1231A3, in a 30 mL bioreactor with stirring blade and demonstrated that undifferentiated hiPSCs were efficiently maintained in this condition (**Figure 5—figure supplement 1A** [↗](#)). These cell lines showed higher cell density in the bioreactor at each passage after 3 days (**Figure 5—figure supplement 1B** [↗](#)). Under these conditions, the concentration of glucose decreased daily (**Figure 5—figure supplement 1C** [↗](#)), whereas that of L-lactic acid increased (**Figure 5—figure supplement 1D** [↗](#)). These results indicate that the cell lines proliferated with active energy consumption. Further, RT-qPCR analysis showed no marked differences in the undifferentiated and differentiation markers expression between hiPSCs cultured in adhesion vs. suspension with PKC β and Wnt inhibitors (**Figure 5—figure supplement 1E** [↗](#) and **F** [↗](#)). Flow cytometric analysis showed that more than 90% of these

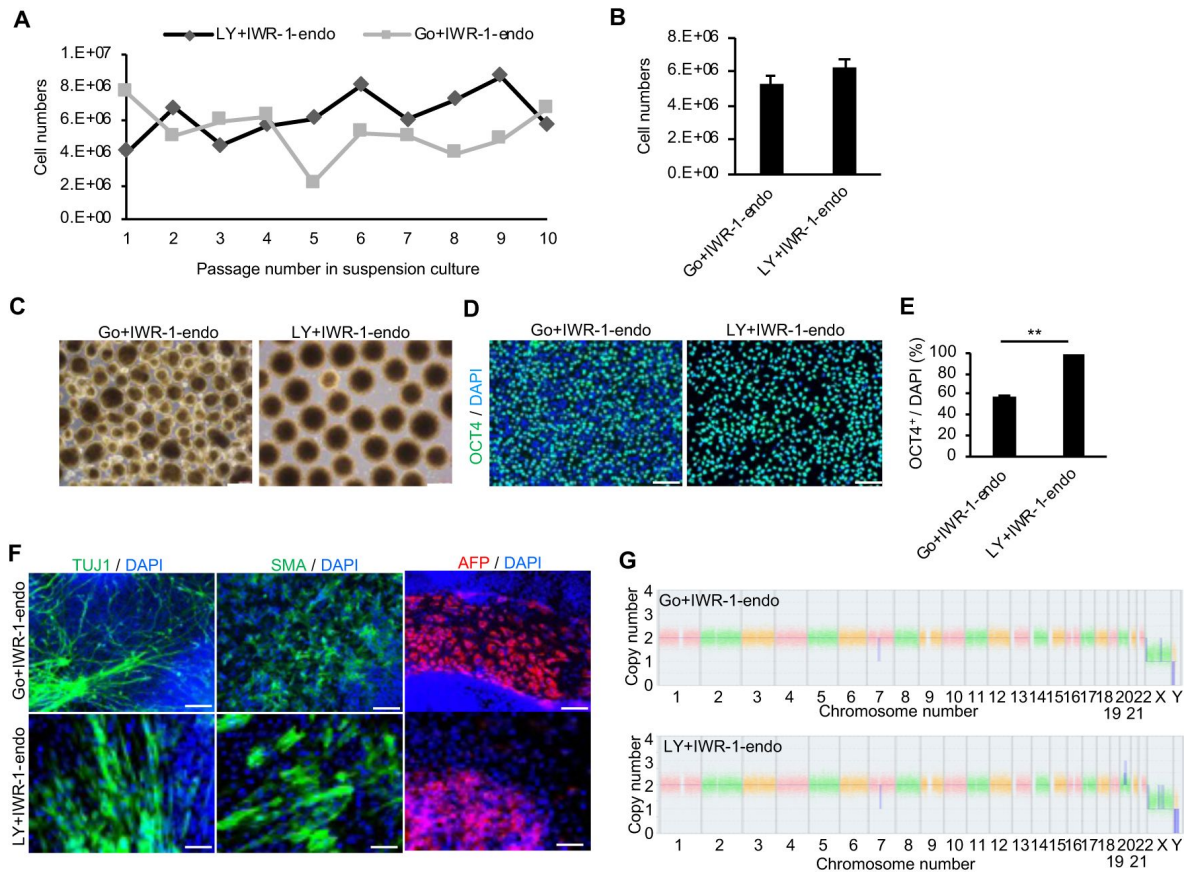


Figure 4.

Long-term suspension culture of hiPSCs is maintained by simultaneous suppression of PKC β and Wnt signals.

(A) The number of hiPSCs at passage during long-term suspension-culture every 5 days. Cell culture was performed in the presence of IWR-1-endo plus Go6983 or LY333531. (B) Bar graph indicating average cell numbers for 10 passages (mean $\pm$ SE). (C) Phase-contrast images of suspension-cultured hiPSCs on passage 10. Scale bars: 400 μ m. (D) Immunocytochemistry of OCT4 on passage 10 samples. Scale bars: 100 μ m. (E) Bar graph showing the percentages of OCT4 positive cells. Values were calculated from randomly selected three regions from immunofluorescence images. Data are presented as mean $\pm$ SE. P-value was statistically analyzed by Student's t-test. (F) Immunocytochemistry of differentiated cells in EBs from suspension-cultured hiPSCs in the presence of IWR-1-endo; and Go6983 or LY333531. Anti-TUJ1, -SMA and -AFP antibodies were used to detect ectoderm, mesoderm and endoderm differentiation, respectively. Scale bars: 100 μ m. (G) Chromosomal copy numbers detected with CGH array analysis (Karyostat assay) of suspension-cultured hiPSCs in the presence of IWR-1-endo; and Go6983 (Upper panel) or LY333531 (Lower panel) at passage 10.

cells were positive for pluripotency marker proteins: NANOG, OCT4, and SOX2 (**Figure 5—figure supplement 1G** [↗](#)). G-band analysis of suspension-cultured hiPSCs after five passages revealed normal karyotype (**Figure 5—figure supplement 1H** [↗](#)). These results demonstrate that undifferentiated hiPSCs were efficiently maintained in the bioreactor using the culture medium supplemented with inhibitors of PKC β and Wnt signaling pathways.

Next, we expanded hiPSCs in a large-scale culture system under perfusion conditions in the presence of IWR-1-endo and LY333531. In this experiment, clinical-grade hiPSC line, Ff-114s04, which is derived from PBMCs of a donor carrying homozygous alleles for major HLA loci (HLA-A, HLA-B, and HLA-DR) was used (Kitano *et al.*, 2022 [↗](#)). Large-scale hiPSCs preparation using a perfusion-culture system with 320 mL bioreactor having stirred-wing was performed in GMP-compliant, clinical grade StemFit AK03N medium containing IWR-1-endo and LY333531 (**Figure 5A** [↗](#)). When the culture was started at 1×10^5 cells/mL in 320 mL medium, hiPSCs proliferated nearly 10-fold after 3 to 4 days and reached approximately 1×10^9 cells in total to produce ~300 stock vials (1×10^6 cells/vial). This large-scale culture was repeated three times (Passages 1, 2, and 3), and hiPSCs obtained at each passage were characterized. These samples showed similar or higher viability (>90%) to that of adherent-culture derived cells (**Figure 5B** [↗](#)). When compared to adherent conditions, these samples in suspension-culture showed a similar or higher proliferation rate (**Figure 5C** [↗](#)). Cells obtained from large-suspension cultures formed typical hiPSC-like colonies, after vials were thawed and seeded in adherent-culture conditions (**Figure 5D** [↗](#)). Flow cytometry detected over 90% of cells positive for pluripotent cell markers expression: TRA-1-60, SSEA4, and OCT4 (**Figure 5E** [↗](#) and **F** [↗](#)). Further, G-band analysis revealed that hiPSCs retained their normal karyotype even after three passages under large-scale suspension conditions (**Figure 5G** [↗](#)). When large-scale suspension-culture derived hiPSCs were incubated in each germ layer-specific differentiation medium for 4 - 7 days, the expression of early differentiation markers for ectoderm (PAX6 and SOX1), mesoderm (T and PDGFRA) and endoderm (SOX17 and CXCR4) was significantly induced (**Figure 5H** [↗](#)). These cells were then directly differentiated into dopaminergic neural progenitors, cardiomyocytes, and hepatocytes to evaluate their differentiation capacity and propensity. There were no differences in the differentiation efficiency toward these lineages (**Figure 5I-M** [↗](#)). Hence, these results indicate that the characteristics and quality of clinical-grade hiPSCs cultured in large-scale suspension conditions in the presence of PKC β and Wnt inhibitors were equivalent to those of hiPSCs maintained under adherent conditions. Taken together, we were successful in mass suspension-culture conditions supplemented with Wnt and PKC β inhibitors.

Single cell sorting and expansion of hiPSC subclones cultured in suspension conditions

We sorted a hiPSC line, 201B7, with TRA-1-60 antibody into individual wells of a 96-well plate and attempted to establish subclones by expanding them with serial passages (**Figure 6A** [↗](#)). By day 28 after this single-cell sorting, we expanded single-cell-sorted hiPSC subclones in suspension culture supplemented with IWR1-endo and LY333531 (**Figure 6B** [↗](#)). On day 28, we examined seven subclones for their cell growth and expression of OCT4 and TRA-1-60. The subclones showed more than three million cells and high ratios of OCT4- and TRA1-60-positive cells (**Figure 6C-E** [↗](#)). These results indicate that we have successfully derived single-cell-cloned sublines in suspension culture.

Direct freeze and thaw of hiPSCs cultured in suspension conditions

We froze a hiPSC line, 201B7, in suspension culture conditions supplemented with IWR1-endo and LY333531. Then, we thawed these frozen vials and directly reseeded the cells in suspension conditions (**Figure 6—figure supplement 1A** [↗](#)). By day 10 after reseeded, we expanded the hiPSCs in suspension culture supplemented with IWR1-endo and LY333531 (**Figure 6—figure**

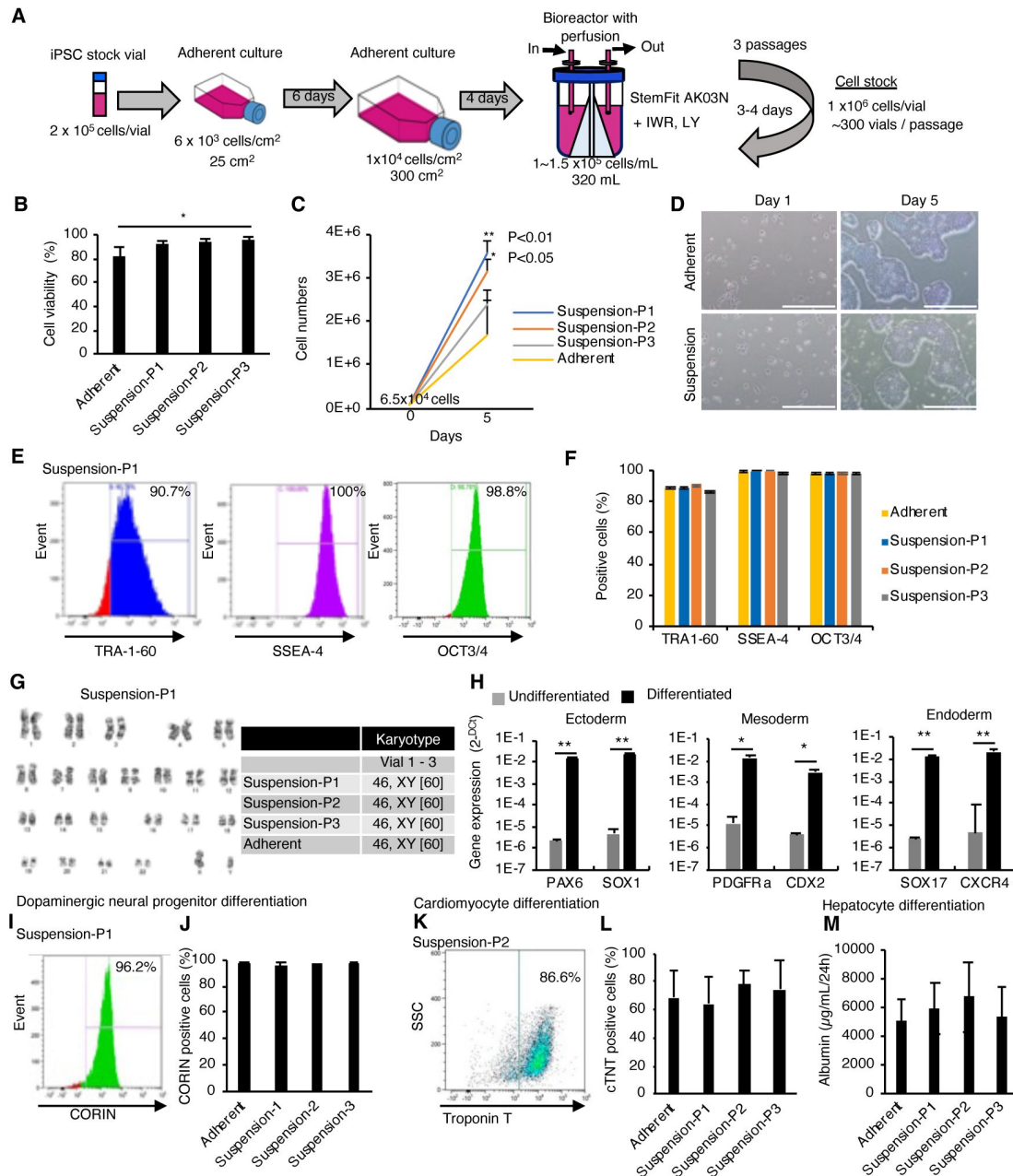


Figure 5.

Mass suspension-culture of clinical-grade hiPSCs in the presence of PKCβ and Wnt signaling inhibitors.

(A) Schematics. (B) Cell viability at seeding. Data are presented as mean ± SE (n=3). P-values were statistically analyzed with Dunnett's multiple comparisons test. (C) Total cell numbers were counted at day 5. Data were presented as mean ± SE (n=3). P-values were statistically analyzed with Dunnett's test. (D) Representative phase-contrast images of these hiPSCs. Scale bar: 1 mm. (E) Representative flow cytometry data of pluripotent markers in these hiPSCs. (F) Quantification of flow cytometry data for the pluripotent markers. Data are presented as mean ± SE (n=3). (G) Karyotypes of these hiPSCs. Left: Representative pictures of G-band analysis. Right: Table of karyotype results (n=3). The numbers in brackets indicate the cell numbers examined. (H) *In vitro* differentiation from hiPSCs was assessed using RT-qPCR (mean ± SE) (n=3). P-value was statistically analyzed by Student's t-test. (I) Representative flow cytometry data for CORIN. (J) Quantification of CORIN-positive cells (mean ± SE, n=3). (K) Representative flow cytometry data for cardiac Troponin T (cTnT). (L) Quantification of cTnT-positive cells (mean ± SE, n=3). (M) Albumin secretion levels of hepatocytes differentiated from hiPSCs (mean ± SE, n=3).

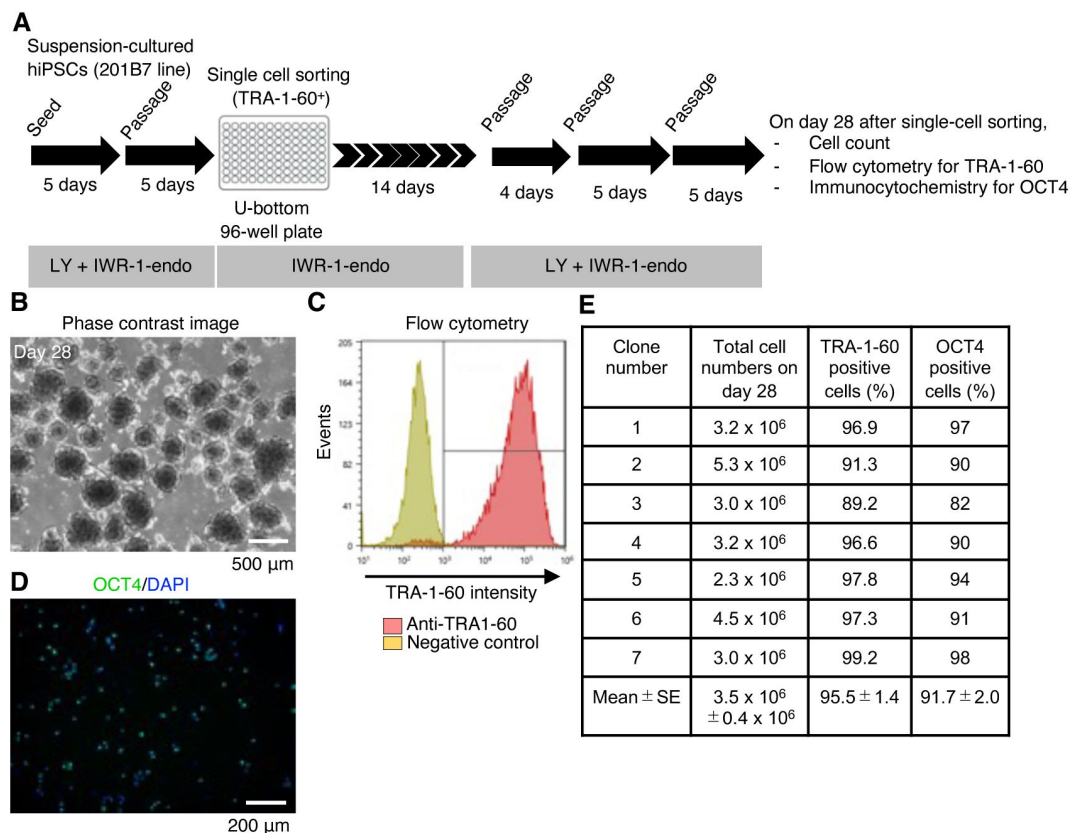


Figure 6.

Establishment of single-cell-sorted hiPSC subclones cultured in suspension conditions supplemented with IWR1-endo and LY333531.

(A) Schematics representing the establishment of single-cell-derived hiPSC subclones from 201B7 line. Single-cell-sorted cells were expanded in the culture medium supplemented with IWR-1-endo and LY333531. Formed colonies were picked on day 14, and expanded by repeating passage every 4-5 days under suspension conditions. Characteristic analysis was performed on day 28 after single-cell-sorting. (B) Phase-contrast images of 201B7 on day 28 after single-cell sorting (Clone #2). Scale bars: 500 μ m. (C) Represented flow cytometry of TRA-1-60 (Clone #2). (D) Represented immunocytochemistry of OCT4 (Clone #2). Scale bars: 200 μ m. (E) Summary of the characterization of single cell-sorted clones. Total cell numbers on day 28, the ratio of TRA-1-60-positive cells (%), and the ratio of OCT4-positive cells (%) are shown (mean $\pm$ SE) (n=7).

supplement 1B [↗](#)). On day 10, we examined three vials for their cell growth and expression of OCT4 and TRA-1-60. The subclones showed more than three million cells and high ratios of OCT4- and TRA1-60-positive cells (**Figure 6—figure supplement 1C-E** [↗](#)). These results indicate that we have successfully frozen and thawed hiPSCs in suspension culture directly.

Together, these results suggest that the suspension culture conditions supplemented with Wnt and PKC β inhibitors are robust in harsh conditions such as single-cell expansion and freezing-and-thaw processes.

Establishment of hiPSC lines in complete suspension-culture conditions supplemented with inhibitors of PKC β and Wnt signaling pathways

For the clinical use of hiPSCs, more secure and efficient establishment of hiPSCs in closed systems is strongly desired. To achieve this, we aimed to establish hiPSCs in suspension-culture conditions. Using PMBCs as a starting material, we generated hiPSCs using a novel replication-defective and persistent Sendai virus vector (SeVdp) infection or episomal vector electroporation, as these methods are well-known for producing transgene-free, clinical-grade hiPSCs (Fusaki *et al*, 2009 [↗](#); Nishimura *et al*, 2011 [↗](#)) (**Figure 7A** [↗](#)). PMBCs were infected with SeVdp carrying OCT4, SOX2, KLF4, and L-MYC. Infected cells were kept cultured by repeating passages every 5 - 6 days. Cell aggregates cultured with IWR1-endo and LY33351 showed uniform spherical structure (**Figure 7B** [↗](#)), and most of the cells were positive for OCT4 (**Figure 7C** [↗](#)) and TRA-1-60 (**Figure 7D** [↗](#)) on day 56. These bulk cells were able to differentiate into three germ layers in an *in vitro* EB formation assay (**Figure 7E** [↗](#)) and in teratomas that were transplanted into immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (**Figure 7F** [↗](#)). These bulk-hiPSCs maintained normal karyotype (**Figure 7G** [↗](#)). By single cell sorting with fluorescent labeled TRA-1-60 antibody, we expanded single-cell-derived clones in suspension-culture conditions. A clone (F-10) showed OCT4 and TRA1-60 expressions (**Figure 7H-J** [↗](#)). In addition, the established clone showed potency to differentiate into derivatives of three germ layers *in vitro* as EBs (**Figure 7K** [↗](#)) and *in vivo* as teratoma transplanted into NSG mice (**Figure. 7L** [↗](#)). A normal karyotype was observed in this clone (**Figure 7M** [↗](#)). SeVdp was nearly extinct in these bulk populations and F-10 clone (**Figure 7N** [↗](#)). These results demonstrate that, we were successful in establishing transgene-free hiPSC lines using SeVdp infection in suspension-culture conditions in the presence of PKC β and Wnt inhibitors.

Furthermore, we were successful in establishing single-cell-derived hiPSC lines using the transfection of episomal plasmid vectors in suspension-culture conditions in the presence of PKC β and Wnt signaling inhibitors. PMBCs were transfected with episomal plasmid vector mix carrying OCT4, SOX2, KLF4, L-MYC, LIN28, and mp53DD and cultured in suspension conditions in the presence or absence of IWR-1-endo and LY33351 with repeated passages to reach enough cell numbers to make cell stocks and characterization (**Figure 7—figure supplement 1A** [↗](#)). After 36 days of culture, 59% double positive cells for TRA-1-60 and SSEA4 were observed in control, whereas 78% were seen in the presence of IWR-1-endo and LY333531 (**Figure 7—figure supplement 1B** [↗](#) and **C** [↗](#)). We performed cell sorting of TRA-1-60 and SSEA4 double positive cells and further repeated 5 more passages in the absence or presence of IWR-1-endo and LY333531. Cell aggregates cultured in culture medium supplemented with IWR-1-endo and LY333531 showed uniform spherical structures (**Figure 7—figure supplement 1D** [↗](#)), and the most of these cells were TRA-1-60 and SSEA4 double positive (**Figure 7—figure supplement 1E** [↗](#)). In contrast, cell aggregates cultured without these inhibitors showed lumpy, heterogeneous shapes, and decreased undifferentiated population. Immunostaining of OCT4 on dissociated cell aggregates resulted in higher OCT4 positive cells in IWR-1-endo and LY333531 treated cells (**Figure 7—figure supplement 1F** [↗](#) and **G** [↗](#)). We further performed single cell sorting of TRA-1-60-positive cells from bulk-cultured populations at 10 passages (**Figure 7—figure supplement 1H** [↗](#) and **I** [↗](#)). We selected 3 clones to be characterized further. These clones showed round spherical

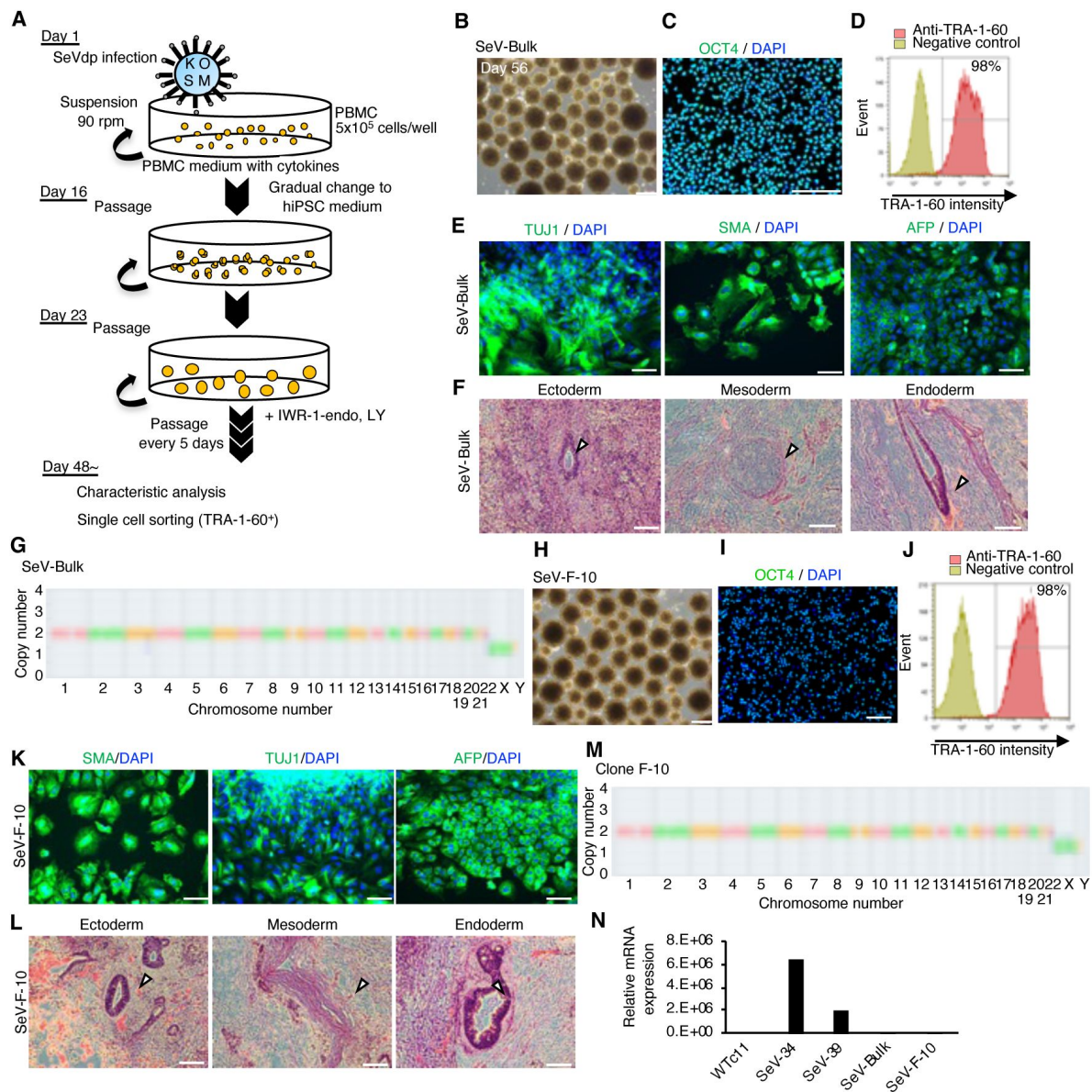


Figure 7.

Establishment of hiPSCs in complete suspension conditions using SeVdp.

(A) Schematics. (B) Phase-contrast images of PBMCs on day 56 after infection. Scale bars: 400 μ m. (C) Immunocytochemistry of OCT4 on bulk-hiPSCs on day 56. Scale bars: 200 μ m. (D) Flow cytometry of TRA-1-60 in bulk-hiPSCs on day 61. (E) Immunocytochemistry of TUJ1, SMA, and AFP on differentiated cells in EBs from bulk-hiPSCs on day 56. Scale bars: 100 μ m. (F) HE staining of teratoma sections derived from bulk-hiPSCs. White arrowheads indicate representative tissue structures derived from ectoderm, mesoderm, and endoderm. Scale bars: 100 μ m. (G) Chromosomal copy numbers detected with CGH array analysis on bulk-hiPSCs. (H) Phase-contrast image of an established hiPSC clone at passage 7. Scale bars: 400 μ m. (I) Immunocytochemistry of established clone, F-10, with anti-OCT4 antibody. Scale bars: 200 μ m. (J) The percentage of TRA-1-60-positive cells with flow cytometry on established clone F-10 line. (K) HE staining of teratoma sections derived from F-10 clone line. The details are the same as in (F). (L) Immunocytochemistry in EBs from established F-10 clone. Scale bars: 100 μ m. (M) Chromosomal copy numbers of F-10 clone line. (N) Residual SeVdp genomic RNA in established hiPSCs with RT-qPCR.

shapes in their aggregates and expression of OCT4, NANOG, and TRA-1-60 proteins (**Figure 7—figure supplement 1J–L** [↗](#)). Pluripotency into three germ layers was confirmed in vitro with EB formation assay (**Figure 7—figure supplement 1M** [↗](#)) and teratoma formation analysis (**Figure 7—figure supplement 1N** [↗](#)). These clones retained a normal karyotype even after a long-culture (**Figure 7—figure supplement 1O** [↗](#)). These results demonstrate that single-cell-derived hiPSC lines were established using the transfection of episomal plasmid vectors in suspension-culture conditions in the presence of PKC β and Wnt signaling inhibitors.

Discussion

In this study, we have developed a series of methods to generate and maintain hiPSCs in suspension conditions. First, we have identified compounds that suppress spontaneous differentiation of hiPSCs in suspension cultures. Based on these findings, we have newly achieved a complete series of culture processes including hiPSC establishment, long-term culture, mass culture, single-cell cloning, and direct freeze and thaw (summarized in **Table 1** [↗](#)). These methods require no special materials including microcarriers or dialysis bags, and are effective with various existing culture media and many hiPSC lines. Therefore, this suspension-culture of hiPSCs is anticipated to have novel industrial applications, which have been never achieved with conventional methods because suspension-culture conditions, which precisely control cellular states, have advantages in terms of mass culture, automation, and safety assurance.

We have identified two compounds as factors that ameliorate the disadvantages of the suspension culture of hiPSCs. Wnt signaling inhibitors specifically inhibited differentiation toward mesendoderm, whereas, the PKC β inhibitor, LY333531, inhibited ectodermal differentiation and, when combined with IWR-1-endo, maintained the undifferentiated nature of hiPSCs with high efficiency. Go6983, a pan-PKC inhibitor, has been used to promote the self-renewal of mammalian PSCs (Dutta *et al*, 2011 [↗](#); Kinehara *et al*, 2013 [↗](#); Rajendran *et al*, 2013 [↗](#); Takashima *et al*, 2014 [↗](#)); however, in these cases, the role of specific isoforms in PSC self-renewal and differentiation are not fully elucidated yet. In this study, we demonstrated that PKC β -specific inhibitor LY333531 as well as Go6983 showed the suppression of neuroectodermal differentiation. Interestingly, these combinations of chemical inhibitors against WNT and PKC signaling pathways are also being used in the induction and maintenance of naive human pluripotent stem cells (Bayerl *et al*, 2021 [↗](#); Bredenkamp *et al*, 2019 [↗](#); Guo *et al*, 2017 [↗](#); Khan *et al*, 2021 [↗](#)). While, in suspension-culture, we did not aim to drive hiPSCs to a naïve state by the use of these combinations of chemical inhibitors conditions, it would be worthwhile to investigate the effect of adaptation of suspension-culture supplemented with PKC β and Wnt signaling inhibitors on the transition of the naïve PSCs types.

We successfully generated hiPSC lines from PBMCs in suspension conditions for the first time by adding the abovementioned compounds to the culture medium. Both transduction methods with SeVdp and episomal plasmid vectors enabled the establishment of hiPSCs under suspension conditions in the presence of IWR-1-endo and LY333531. Clonal expansion of hiPSCs was also performed by single-cell sorting with flow cytometry and were successfully characterized for their self-renewal, pluripotency, and genomic integrity. These results indicate that hiPSC lines generated in suspension conditions have the same quality in terms of their pluripotency, self-renewal, and genomic integrity as those generated with conventional adherent conditions. Precise control of hiPSC status in suspension culture could pave the way for their stable and automated clinical application toward autologous cell therapy of hiPSCs.

Methods

Human subjects

The generation and use of hiPSCs were approved by the Ethics Committee of RIKEN BioResource Research Center, Graduate School of Medicine of Kyoto University, and KANEKA CORPORATION.

Animal experiments

All animal experiments were approved by the Animal Experimentation Committee of the RIKEN Tsukuba branch and were performed according to the committee's guiding principles and the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health.

Data availability

All data are available in the main text or the supplementary materials. RNA-seq data are available in the Gene Expression Omnibus as GSE222833. All the data supporting the findings of this study are available from the lead contact upon reasonable request. Source data is provided with this paper. This paper does not report original code.

HiPSCs cultured in adherent conditions

WTC11 (GM25256 from Coriell Institute) (Hayashi *et al.*, 2016 [DOI](#)), 201B7 (HPS0063 from RIKEN Cell Bank) (Takahashi *et al.*, 2007 [DOI](#)), 454E2 (HPS0077 from RIKEN Cell Bank) (Okita *et al.*, 2011 [DOI](#)), 1383D6 (HPS1006 from RIKEN Cell Bank) (Okita *et al.*, 2011 [DOI](#)), 1231A3 (HPS0381 from RIKEN Cell Bank) (Okita *et al.*, 2011 [DOI](#)), and Ff-I14s04 (CiRA foundation, Kyoto University) (Kitano *et al.*, 2022 [DOI](#)) were used as healthy-donor hiPSC lines. With tdTomato-reporter hiPSC lines, we generated knock-in hiPSC lines for PAX6-TEZ (constructed from 454E2 line) and SOX17-TEZ (constructed from 1383D6 line) using CRISPR-Cas9 genome editing (Tsukamoto *et al.*, 2021 [DOI](#)) (Figure. S1A). These hiPSCs were cultured in StemFit AK02N medium (Cat#AK02N, Ajinomoto, Co., Ltd., Tokyo, Japan). Medium change was performed every day and passaged at 80%–90% confluency after 6–7 days of culture. At passage, PBS/EDTA solution (0.5 mM, Cat#06894-14, Nacalai Tesque) was used to dissociate hiPSC colonies, and these cells were seeded at a density of 2,500 cells/cm². 10 μ M Y-27632 (Cat#HY-10071, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 0.25 μ g/cm² iMatrix-511 silk (Cat#892021, Matrixome Inc, Osaka, Japan) were added to the culture dish on seeding day.

Suspension-culture of hiPSCs

Suspension culture with rotation at 90 rpm was performed with a plate shaker (Cat#WB-101SRC, WAKENBTECH, Kyoto, Japan or #0081704-000, TAITEC, Tokyo, Japan) installed in a CO₂ incubator (Cat#Steri-Cycle i160, Thermo Fisher Scientific, MA, USA) and operated under high humidity continuously during the whole culture period. To start the culture hiPSCs in suspension conditions, 4×10^5 cells were seeded in one well of a low-attachment 6-well plate (Cat#MS-80060, Sumitomo Bakelite, Tokyo, Japan) with 4 mL of StemFit AK02N medium, StemFitAK03N (Cat#AK03N, Ajinomoto, Co., Ltd., Tokyo, Japan), or StemScale PSC suspension medium (A4965001, Thermo Fisher Scientific, MA, USA) supplemented with 10 μ M Y-27632. This plate was placed onto the plate shaker in the CO₂ incubator. The medium without Y-27632 was changed every day unless otherwise specified. On days 3–5, the hiPSC aggregates were dissociated with Accutase (Cat#12679-54, Nacalai Tesque) at 37°C for 10 min and used for the next suspension-culture. This passage was performed every 5 days unless otherwise specified. To screen for factors that inhibit spontaneous differentiation of hiPSCs, chemicals or recombinant proteins were added to the culture medium (listed in **Supplementary Table S1** [DOI](#)).

Bioreactor culture of hiPSCs

A frozen stock of hiPSCs was pre-cultured twice in adherent conditions to prepare enough cell numbers. To prepare enough hiPSCs to start large-scale culture, hiPSCs were pre-cultured in iMatrix-511MG (Cat#892 005, Matrixome, Inc, Osaka, Japan) or Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat#A14700, Thermo Fisher Scientific, MA, USA)-coated cell culture flasks with StemFit AK03N medium including 20 μM IWR-1-endo and 1 μM LY333531. Perfusion-culture was started with $3.2 - 4.8 \times 10^7$ cells in 320 mL of StemFit AK03N medium (Cat#AK03N, Ajinomoto, Tokyo, Japan) with IWR-1-endo and LY333531. The reactor system BioFlo320 (Eppendorf, Hamburg, Germany) was used for large-scale hiPSCs culture. To maintain the lactate concentration below a certain level and to regulate the pH, the culture was carried out by increasing the amount of medium perfusion per unit time in accordance with the cell proliferation transition. To prevent pH decrease, CO_2 concentration was regulated by feedback control in the reactor system. After 3-4 days of culture, the formed hiPSC aggregates were dissociated with TrypLE SELECT (Cat#A12859-01, Thermo Fisher Scientific, MA, USA) collected for making cell stocks (~300 tubes). This perfusion culture was repeated three times (P1, P2, and P3) and the cells were prepared at each expansion step.

HiPSC generation in suspension culture conditions

HiPSCs were generated from healthy donor-derived PMBCs (Precision for Medicine). Thawed PMBCs from a vial containing around 1×10^7 cells were pre-cultured in one well of low attachment 6-well plate including 4 mL of StemSpan-AOF (Cat#ST100-0130, STEMCELL Technologies, Vancouver, Canada) supplemented with recombinant human IL-6 (100 ng/mL), IL-3 (10 ng/mL), SCF (300 ng/mL), TPO (300 ng/mL), and FLT3 ligand (300 ng/mL) (all from FUJIFILM Wako Pure Chemical Corporation, Tokyo Japan). After 24 h of incubation with continuous stirring at 37°C and 5% CO_2 , PMBCs were spun down with centrifugation at $200 \times g$ for 10 min at low deceleration speed and resuspended in StemSpan ACF for cell counting. 2.5×10^6 cells of PMBCs were centrifuged at $200 \times g$ for 10 min with low deceleration speed, and electroporated using Nucleofector 2b device (Lonza, Basel, Switzerland) with iPS cell Generation Episomal vector Mix (Takara Bio, Shiga, Japan) and Amara Human CD34⁺ Cell Nucleofector kit (Lonza, Basel, Switzerland) according to manufacturer's protocol. 5×10^5 of electroporated cells were seeded in one well of a low attachment 6-well plate in 4 mL StemSpan ACF with the cytokines mentioned above. Suspension-culture was performed with continuous agitation at 90 rpm. Stem span ACF medium were gradually replaced with StemFit AK02N medium. Formed cell aggregates were passaged with Accutase on day 16 in the presence of 10 μM Y27632, and suspension-culture was continued until the cell numbers reached a sufficient amount for characterization. 10 μM IWR-1-endo and 1 μM LY333531 were added from day 3.

SeVdp infection was performed with Sendai Reprogramming Kit (CytoTune EX-iPS of virus solution, ID Pharma, Tsukuba, Japan) according to the manufacturer's protocol with some modifications. Briefly, pre-cultured 1×10^6 PMBCs were centrifuged at $200 \times g$ for 10 min, with low deceleration speed, and resuspended in 2 mL of StemSpan ACF with cytokines. PMBCs were gently mixed with 2 mL of virus solution prepared at MOI = 5 per 1×10^6 cells. 5×10^5 infected cells were seeded in one well of low-attachment 6-well plate at a total volume of 4 mL with StemSpan ACF plus cytokines. Suspension-culture was initiated with continuous agitation at 90 rpm. Stem span ACFs were gradually replaced with StemFit AK02N as mentioned above, and the cell aggregates were passaged with Accutase on day 16 in the presence of 10 μM Y-27632. Suspension-culture and passages continued until cell reached a sufficient number for characterization. 10 μM IWR-1-endo and 1 μM LY333531 were added from day 23.

Three germ layer differentiation *in vitro*

For Embryoid body (EB) formation assay, suspension-cultured hiPSC lines were dissociated with Accutase and 1.0×10^4 cells were seeded in each well of an EZ-BindShut 96-well-V plate (Cat#4420-800SP AGC TECHNO GLASS CO., LTD, Shizuoka, Japan) with 100 μL Stem Fit AK02N medium

supplemented with 10 μ M Y-27632. Before culture, the 96-well-V plate was centrifuged at 200 x g for 3 min for efficient cell mass formation. The next day, the culture medium was switched to DMEM high Glucose (Cat#08458-16, Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (here after referred as EB medium). On day 8, EBs were transferred into 0.1% (w/v) Gelatin Solution (Cat#190–15805, FUJIFILM Wako Pure Chemical Corporation, Tokyo Japan)-coated 12-well plate and further cultured in EB medium for another 8 days. The medium was changed every day. EBs were fixed with 4% paraformaldehyde in Dulbecco's Phosphate Buffered Saline (D-PBS) (Cat#14249-24, Nacalai tesque, Kyoto, Japan) for 10 min at room temperature and used for immunostaining. Differentiation was validated by immunostaining against each germ layer markers.

The differentiation potency of suspension-cultured hiPSCs toward three germ layers was also evaluated by culturing in germ layer specific differentiation medium. For ectodermal differentiation, suspension-cultured hiPSCs spheroids were cultured with Stem Fit AK02N without C medium supplemented with 10 μ M SB431542 (Cat#198-16543, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) and 10 μ M DMH1 (Cat#041-33881FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) for 7 days. For mesodermal differentiation, suspension-cultured hiPSCs spheroids were cultured with RPMI1640 medium (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) supplemented with B-27 supplement, minus insulin (A1895601, Thermo Fisher Scientific, MO, USA) and 7.5 μ M CHIR99021 (Cat#034-23103, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) for 1 day, and continuously with RPMI1640 medium supplemented with B-27 supplement for 2 days. For endodermal differentiation, suspension-cultured hiPSCs spheroids were cultured with RPMI1640 medium supplemented with 1 mM Sodium pyruvate (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), 1 x NEAA (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), 80 ng/mL Activin A (R&D Systems, MN, USA), 55 μ M 2-mercaptethanol (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan), 50 ng/mL FGF2 (R&D Systems, MN, USA), 20 ng/mL BMP4, and 3 μ M CHIR99021 for 2 days, and continuously with RPMI1640 medium supplemented with 1 mM Sodium pyruvate, 1x NEAA, 80 ng/mL Activin A, 55 μ M 2-mercaptethanol, and 0.5% Knockout serum replacement (KSR; Thermo Fisher Scientific, MO, USA) for 2 days.

Neuroectoderm and endoderm differentiation of reporter hiPSC lines

Reporter hiPSC lines, PAX6-TEZ and SOX17-TEZ were used as positive controls for in vitro neuroectoderm and endoderm differentiation, respectively. PAX6-TEZ was seeded in each well of a 24 well-plate coated with 0.25 μ g/cm² iMatrix-511 silk with 1 mL StemFit AK02N medium supplemented with 10 μ M Y-27632. Next day, the culture medium was switched to Stem Fit AK02N medium without supplement C, instead of containing 10 μ M SB431542 and 10 μ M DMH1. SOX17-TEZ was seeded in each well of a 24 well-plate (same as above), and on the next day, the culture medium was switched to StemFit AK02N medium without supplement C instead of containing 3 μ M CHIR99021 and 10 ng/mL Activin A (Cat#014-23961, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan). The medium was changed every day. On day 7, tdTomato expression was observed under all-in-one fluorescent microscope (BZ-X800; KEYENCE).

Cardiomyocyte differentiation

Cardiomyocyte differentiation was performed according to a modified method described previously (Funakoshi *et al.*, 2016 [DOI](#)). Both large-scale suspension cultured hiPSCs and typical adherent-cultured hiPSC were collected by centrifugation after dissociation into single cell with TrypLE Select. The cells were suspended in 1.5 mL cardiomyocyte differentiation media (CDM), consisting of StemPro34 medium (Thermo Fisher Scientific) supplemented with 2 mM Glutamax (Thermo Fisher Scientific), 50 μ g/mL ascorbic acid, 4 $\times$ 10⁻⁴ M monothioglycerol (Sigma-Aldrich), 150 μ g/mL transferrin (Roche), and with Matrigel (Cat#354277, Corning, NY, USA), 10 μ M Y-27632 and 2 ng/mL human recombinant BMP4 (R&D Systems) and then cultured in ultra-low attachment

6 well plate (Corning) in 5%CO₂/5% O₂. After 24 h, 1.5 mL CDM with 6 ng/mL human recombinant activin A (R&D Systems) and 5 ng/mL bFGF (R&D Systems) were added into the wells. After 3 days, medium was changed to 3 mL CDM with 10 ng/mL VEGF (R&D Systems), SB431542 (Sigma-Aldrich), Dorsomorphin (Sigma-Aldrich) and 1 μM IWP-3 (Stemgent). After 7 days, medium was changed to 3 mL CDM with 10 ng/mL VEGF (R&D Systems). The medium was changed every 2 days. The cells were collected at 15 days after differentiation and analyzed by flow cytometry.

Dopaminergic progenitor cells differentiation

Dopaminergic progenitor cells differentiation was performed according to a modified method described previously (Doi *et al.*, 2020 [DOI](#)). Both large-scale suspension cultured hiPSCs and typical adherent-cultured hiPSC were collected by centrifugation after dissociation into single cell with TrypLE Select. The cells were suspended in 1 mL dopaminergic progenitor cells differentiation medium-1 (DPM-1), consisting of Glasgow's minimum essential medium (Thermo Fisher) supplemented with 8% knockout serum replacement (Thermo Fisher Scientific), 1% MEM Non-Essential Amino Acids Solution (Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (FujiFilm Wako) and 100 nM LDN193189 (Stemgent), and were then seeded in iMatrix-coated 24-well plates at a density of 1×10^6 cells/well with 10 μM Y-27632 and 500 nM A-83-01 (FujiFilm Wako). After 1 and 2 days, medium was changed to DPM-1 with 500 nM A-83-01, 100 ng/mL recombinant human FGF8 (FujiFilm Wako), and 2 μM purmorphamine (FujiFilm Wako). After 3 to 6 days, medium was changed every day to DPM-1 with 500 nM A-83-01, 100 ng/mL recombinant human FGF8, 2 μM purmorphamine, and 3 μM CHIR99021 (FujiFilm Wako). After 7 to 11 days, medium was changed every day to DPM-1 with 3 μM CHIR99021. On differentiation day 12, cells were dissociated using TrypLE select and suspended in the dopaminergic progenitor cells differentiation medium-2 (DPM-2), consisting Neurobasal medium (Thermo Fisher Scientific), 2% B27 supplement (without Vitamin A, Thermo Fisher), 1% GlutaMAX (Thermo Fisher Scientific), 10 ng /mL human recombinant glial cell-derived neurotrophic factor (FujiFilm Wako), 200 mM ascorbic acid, 20 ng / mL human recombinant brain-derived neurotrophic factor (FujiFilm Wako), and 400 μM dibutyryl cAMP (FujiFilm Wako), and were then plated on U-shaped 96-well plates (Thermo Fisher Scientific) at a density of 2×10^4 cells/150 μL/well with 10 μM Y-27632. After 15 to 26 days, half of medium was changed every 2 days to DPM-2. Dopaminergic progenitor cells differentiation efficiency was analyzed by flow cytometry at 12 days after differentiation. On differentiation day 12, cells were labeled with anti-CORIN antibody (Clone 5B6, Sigma-Aldrich) and secondary antibody (A11001, Thermo Fisher).

Hepatocyte differentiation

Hepatocyte differentiation was performed according to a modified method described previously (Si-Tayeb *et al.*, 2010 [DOI](#)). Both large-scale suspension-cultured and typical adherent-cultured hiPSCs were collected by centrifugation after dissociation into single cell with TrypLE Select. These hiPSCs were cultured on Matrigel-coated 6-well plates until 80% confluency with StemFit medium (Ajinomoto). To initiate differentiation, the medium was changed to 1 mL Hepatocyte differentiation medium (HDM) consisting of RPMI1640 medium (Thermo Fisher) supplemented with 1× GlutaMax (Thermo Fisher) and 1× B27 Supplement minus vitamin A, with the addition of 100 ng/mL Activin A (R&D Systems). After 1 to 3 days, medium was changed every day to HDM with 100 ng/mL Activin A. After 4 to 8 days, medium was changed every day to HDM with 20 ng/mL human recombinant BMP-4 (R&D Systems) and 20 ng/mL human recombinant FGF-4 (R&D Systems). After 9 to 13 days, medium was changed every day to HDM with 20 ng/mL human recombinant HGF (R&D Systems). After 14 to 24 days, medium was changed every 2 days to Hepatocyte Culture Medium (HCM; Lonza) with 20 ng/mL human recombinant Oncostatin M (R&D Systems). On differentiation day 25, the albumin concentration in the culture supernatant was determined by ELISA using an anti-human albumin antibody (Bethyl Laboratories, Catalog # E88-129).

Teratoma formation

Suspension-cultured hiPSCs were dissociated with Accutase and then resuspended with 1 mL of StemFit AK02N medium supplemented with 10 μ M Y-27632. 1×10^6 cells were collected by centrifugation at $200 \times g$ for 3 min and suspended in ice-cold 50% Matrigel solution: StemFit AK02N with 10 μ M Y-27632 = 1:1. Cells were injected into testis or leg of NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, USA) using an 18-G needle. Two to three months later, teratomas were collected and fixed with 4% paraformaldehyde in D-PBS. Paraffin-embedded sectioning and hematoxylin-eosin (HE) staining was performed. Three germ layer derivatives were observed under CKX53 microscope with a DP22 camera and CellSens software (EVIDENT, Tokyo, Japan).

Karyotyping

For virtual karyotyping, genomic DNA (gDNA) was extracted from hiPSCs using a DNeasy Blood & Tissue Kit (Cat#69504, QIAGEN, Hülsterweg, Netherland) and was used for microarray assay. Virtual karyotyping was performed with GeneChip Scanner System 3000 using Karyostat Assay arrays (Cat#905403, both from Thermo Fisher Scientific, MA, US) according to the manufacturer's protocol. Data were analyzed using the Chromosome Analysis Suite (ChAS) and Affymetrix GeneChip Command Console software programs.

G-band analysis was performed using the common Giemsa staining method with hiPSCs fixed by Carnoy's fixative (3:1 ratio of methanol: glacial acetic acid).

qRT-PCR

Total RNA was extracted with a FastGene RNA premium kit (Cat#FG-81250, NIPPON Genetics, Tokyo, Japan) and used for reverse transcription reaction. cDNA was synthesized by using a ReverTra Ace qPCR RT kit (Cat#FSQ-101, TOYOBO, Osaka, Japan) with random primers. Real-Time qPCR reactions were performed with a QuantStudio 3 System (Thermo Fisher Scientific) using THUNDERBIRD Probe qPCR Mix (Cat#QPS-101, TOYOBO) with TaqMan probes (listed in **Supplementary Table 2** [↗](#)) (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene expression was described as the fold change relative to the control sample value ($\Delta\Delta C_t$ method) after normalization to the corresponding GAPDH or β -Actin values, unless otherwise specified. For the residual SeV detection, data were normalized to the expression of GAPDH and displayed as relative fold increase to hiPSC line established with episomal vector (WTc11). SeVdp-infected fibroblasts on passage 1 (SeV-34 and -39) were used as positive controls.

Immunocytochemistry

Immunocytochemistry was performed on adherent cells. Suspension-cultured hiPSCs were dissociated with Accutase and transiently cultured as adherent in iMatrix-coated culture dishes for 3 h before immunocytochemistry. The cells were fixed with 4% paraformaldehyde in D-PBS for 10 min at room temperature, then permeabilized in D-PBS containing 0.1% Triton X-100 for another 10 min. Cells were incubated with primary antibodies in D-PBS containing 0.1% Bovine Serum Albumin (BSA; Cat#017-22231, FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) overnight at 4 °C. The secondary antibodies were incubated for 1 hour at room temperature in D-PBS containing 0.1% BSA. Fluoro-KEEPER Antifade Reagent, Non-Hardening Type with DAPI (Cat#12745-74, Nacalai tesque, Kyoto, Japan) was used for nuclear counterstaining. Fluorescence images were taken with an all-in-one fluorescent microscope (BZ-X800; KEYENCE, Osaka, Japan). The primary and secondary antibodies used in this study are listed in **Supplementary Tables 3** [↗](#) and **4** [↗](#), respectively.

Automatic capillary Western blot (Simple Western Assays)

To detect the expression of PAX6 or SOX17, adherent- or suspension-cultured PAX6-TEZ or SOX17-TEZ hiPSCs were collected on day 10 (Passage 2). The cell lysate was prepared from 1×10^6 cells with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer solution without 2-ME (2x) (Cat#30567-12, Nacalai Tesque) supplemented with 100 mM Dithiothreitol (DTT) (Cat#14130-41, Nacalai Tesque). To detect phosphorylated PKC β , cell lysates were collected on day 5 of suspension-cultured hiPSCs with extraction buffer mentioned above, containing 1% phosphatase inhibitor cocktail (Cat#07574-61, Nacalai Tesque). Samples were denatured at 95°C for 5 min. Western blotting was performed with a capillary automatic western blotting device (Simple Western, Wes; Bio-Techne, CA, USA). Jes/Wes 12- to 230-kDa separation module for Wes, 8 $\times$ 25 capillary cartridges (Cat#SM-W004, ProteinSimple), and Anti-Rabbit/Goat/Mouse Detection Module kit (Cat#DM-002/DM-001/DM/006, ProteinSimple) were used. Preparation of reagents and sample loading were conducted according to the manufacturer's instructions. The data were analyzed and quantified by compass for simple Western software program (ProteinSimple). GAPDH was used as the reference for normalization. The primary antibodies used in this study are listed in **Supplementary Table 3** [↗](#).

Flow cytometry

Flow cytometry of self-renewal markers, TRA-1-60 and SSEA4 was performed as described in our previous study. Briefly, adherent- or suspension-cultured hiPSCs were dissociated with 0.5 mM EDTA solution in D-PBS or Accutase, respectively. Dissociated cells (0.5 or 1×10^6 cells) were collected and centrifuged at $200 \times g$ for 3 minutes and resuspended in 500 or 1000 μ L PBS containing 0.1% BSA and 0.5 mM EDTA, then incubated with or without anti-TRA-60 antibody or anti-SSEA4 antibody for 1 hour at 4 °C under oblique light conditions. After washing with PBS, cells were resuspended in 500 mL D-PBS containing 0.1% BSA and 0.5 mM EDTA, and filtrated with 35-mm cell strainer (Cat#352235, FALCON, Thermo Fisher Scientific). For PAX6-TEZ and SOX17-TEZ hiPSC lines, adherent- or suspension-cultured hiPSCs were dissociated into single cells and collected cells were resuspended in 500 mL PBS containing 0.1% BSA and 0.5 mM EDTA. After passing through the 35-mm cell strainer, cells were analyzed for tdTomato. The primary and secondary antibodies used in this study are listed in **Supplementary Table 3** [↗](#) and **4** [↗](#), respectively. Flow cytometry was performed with SH800 cell Sorter (SONY, Tokyo, Japan).

RNA-Seq

Total RNA was extracted using the FastGene RNA premium kit, and strand-specific library preparation was performed. The prepared library was sequenced using a NovaSeq6000 (Illumina, Inc, CA, USA). Sequencing was performed in a 150 bp $\times$ 2 paired-end configuration with a data output of about 6 Gb per sample ($\sim$ 20 million paired reads). Library preparation and sequencing was performed in GENEWIZ (Azenta, MA, USA). To identify differentially regulated genes, sequencing data was analyzed with a CLC Genomics Workbench (QIAGEN, Hulsterweg, the Netherlands) and R package, edgeR (v3.30.3) in R language (v 4.1.0). An MA plot (log2 fold change versus mean average expression) comparing transcriptomes between suspension- and adherent-culture from RNA-seq data. Transcripts with log2 fold change ≥ 2 or ≤ -2 (FDR <0.01) are highlighted with red and blue dots, respectively. The extracted genes were analyzed for gene ontology enrichment and biological pathways using the R package, clusterProfiler (v3.16.1). GSEA was also performed using clusterProfiler. An enrichment map was used to visualize the GSEA results with enrichment score (ES) and false discovery rate (FDR) values. Software used for the analysis is listed in **Supplementary Table 5** [↗](#).

Statistical analysis

Statistical analysis was performed using Student's t-test or Dunnett's test for multiple comparisons with a single control condition. P values < 0.05 were considered statistically significant. *, ** or *** in the graphs indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

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Author Contributions

Conceptualization: YHA, Methodology: MMT, SK, CW, KT, MI, YHA, Investigation: MMT, SK, YHE, TW, TS, YA, KT, MI, MN, MU, TMK, MN, KN, YN, YHA, Visualization: MMT, SK, TS, YH, Funding acquisition: MMT, TN, YK, YHA, Project administration: MMT, TN, YK, YHA, Supervision: MMT, TN, YN, NT, YK, YHA, Writing – original draft: MMT, KS, MT, YHA, Writing – review & editing: MMT, YHA

Competing Interest Statement

Y. HA received collaborative research grant from KANEKA corporation during this study. S. K., K.T., M. I., Y.K., and T.N. were employed with KANEKA corporation during this study. M.M.T., S.K. K.T., M. I., Y.K., and Y.HA. are inventors of patents arising from this work (WO/2021/162090 and PCT/JP2020/005255). The other authors declare no competing interests.

Classification

Applied Biological Sciences, Cell Biology, and Developmental Biology in BIOLOGICAL SCIENCES

Figures and Tables

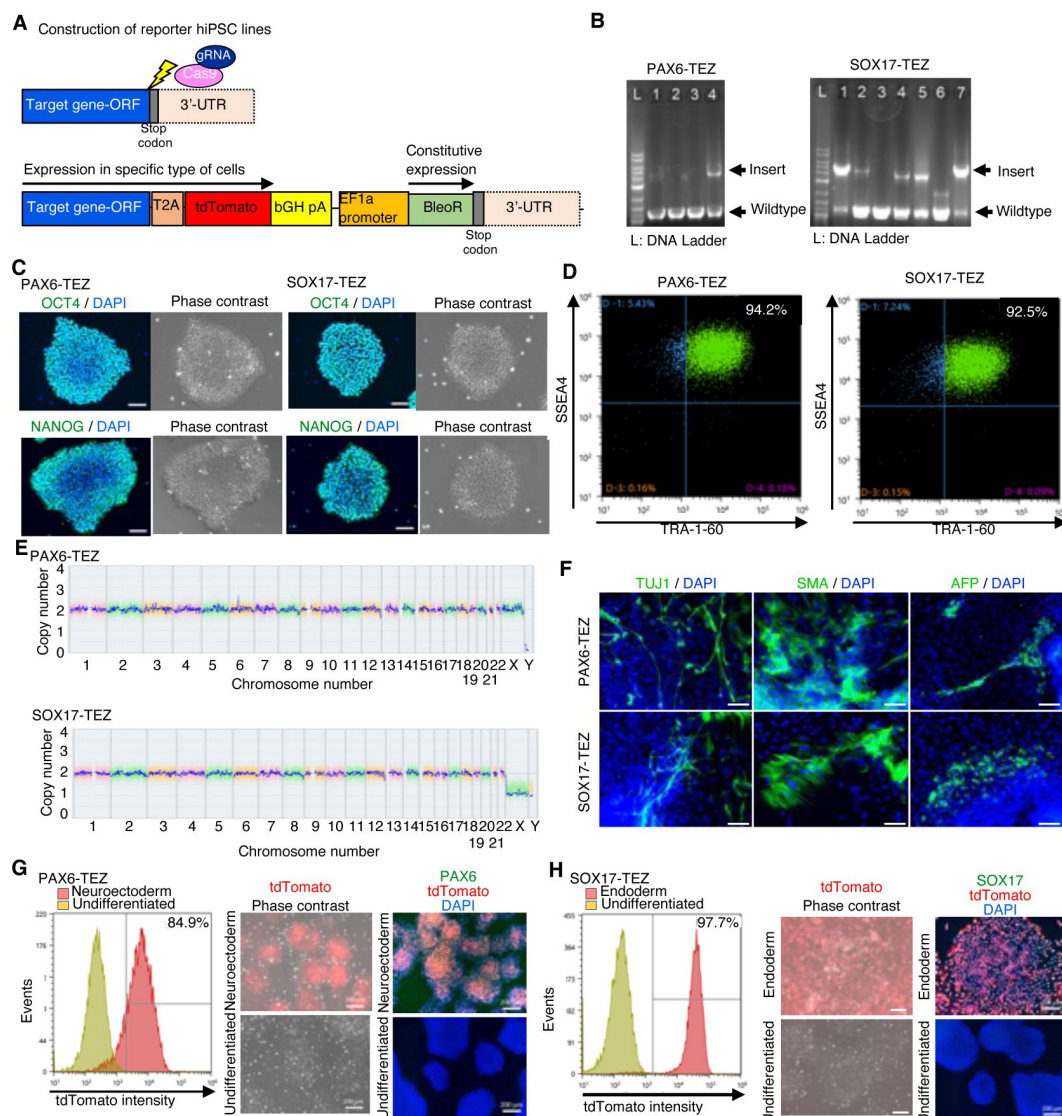


Figure 1—figure supplement 1.

Characterization of PAX6-tdTomato and SOX17-tdTomato reporter lines.

(A) Schematic of construction of reporter hiPSC lines. CRISPR/Cas9 was employed to knock-in the 2A-tdTomato gene and EF1a promoter-driven Zeocin (Bleomycin) resistance gene sequence (TEZ) to the 3' terminal of SOX17 or PAX6 open reading frame (ORF), respectively. (B) Genotyping of selected clones by genomic PCR. Arrows indicate alleles of wildtype or insertion in the PAX6 (left panel) or SOX17 (right panel) genes. (C) Phase-contrast images of representative colonies and immunocytochemistry of pluripotency markers: OCT4 and NANOG. Left panels: PAX6-TEZ. Right panels: SOX17-TEZ. (D) Flow cytometry of cell surface markers: TRA-1-60 and SSEA4. Left: PAX6-TEZ. Right: SOX17-TEZ. (E) Chromosomal copy numbers detected with CGH array analysis of PAX6-TEZ (top) and SOX17-TEZ (bottom). (F) Immunocytochemistry of differentiated cells in EBs from established bulk-hiPSCs on day 56. Anti-TUJ1, -SMA and -AFP antibodies were used. Scale bars: 100 μ m. (G) Differentiation potency of PAX6-TEZ in the neural ectoderm. Left: Flow cytometry of tdTomato using PAX6-TEZ cultured in neuroectodermal differentiation medium for 7 days. Middle: Overlay images of phase-contrast and tdTomato fluorescence of PAX6-TEZ cultured in neuroectodermal differentiation medium (upper) or maintenance medium (lower). Right: Overlay images of tdTomato fluorescence and endogenous PAX6 protein detected with immunocytochemistry with anti-PAX6 antibody. (H) Differentiation potency of SOX17-TEZ in the endoderm. Left: Flow cytometry of tdTomato using SOX17-TEZ cultured in endodermal differentiation medium for 7 days. Middle: Overlay images of phase-contrast and tdTomato fluorescence of SOX17-TEZ cultured in endoderm differentiation medium (upper) or maintenance medium (lower). Right: Overlay images of tdTomato and endogenous SOX17 protein detected by immunocytochemistry with anti-SOX17 antibody.

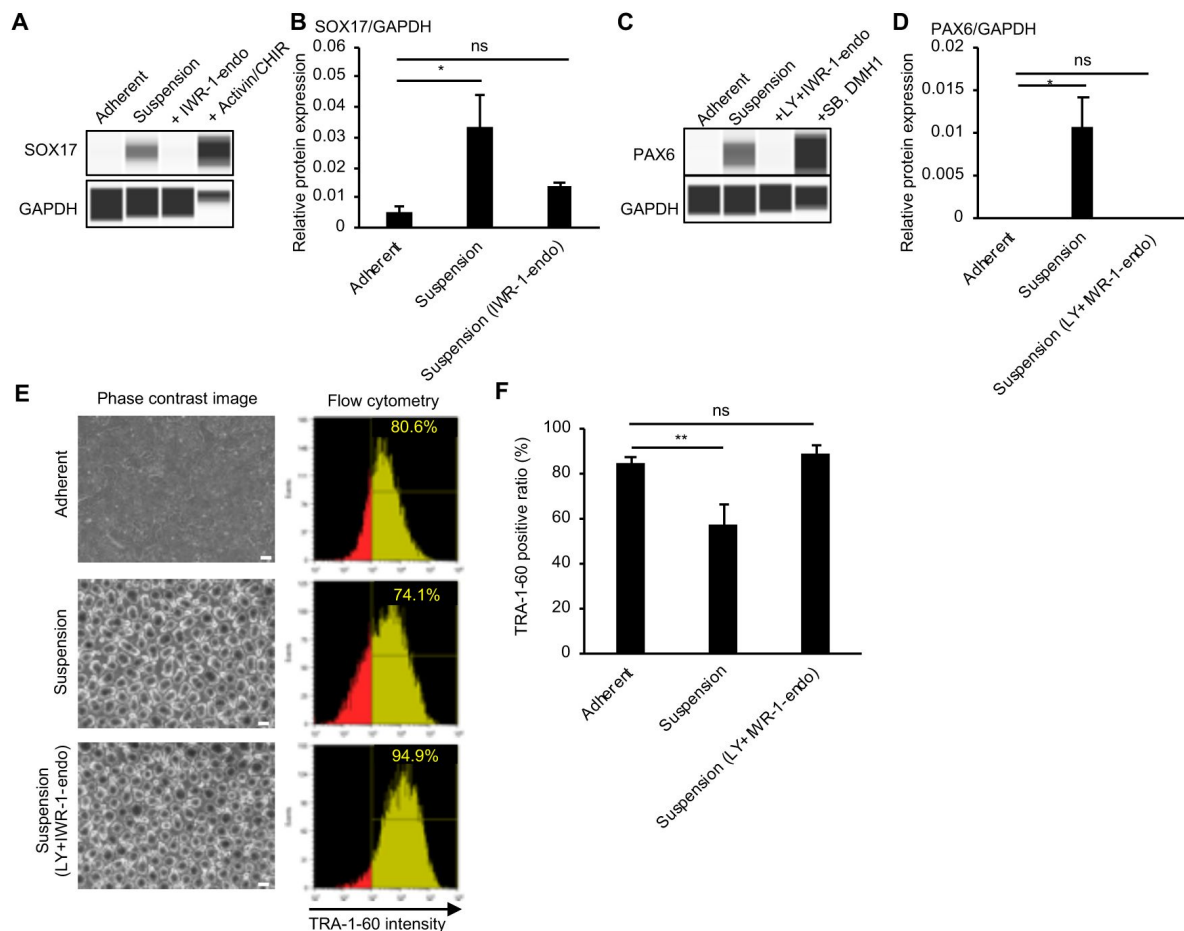


Figure 2—figure supplement 1.

Effects of PKC β and IWR-1-endo on suspension-cultured hiPSCs at the protein level.

(A) Simple western blotting of adherent- or suspension-cultured hiPSCs (SOX17-TEZ) in the presence or absence of IWR-1-endo. SOX17-TEZ cultured in endoderm differentiation medium (Actinin A + CHIR) was used as a positive control for SOX17 expression. (B) Quantification of SOX17 expression. The protein expression was normalized to GAPDH. Data are presented as mean $\pm$ SE (n=3). (C) Simple western blotting of adherent- or suspension-cultured hiPSCs (PAX6-TEZ) in the presence or absence of IWR-1-endo and LY333531. PAX6-TEZ cultured in neurectoderm differentiation medium (SB431542+DMH1) was used as the positive control for PAX6 expression. (D) Quantification of expression of PAX6. The protein expression was normalized to GAPDH. Data are presented as mean $\pm$ SE (n=3). (E) Phase-contrast images and representative flow cytometry data of pluripotency marker, TRA-1-60, in adherent- or suspension-cultured hiPSCs (1383D6 line) on day10 (Passage 2). Suspension-culture was performed in the presence or absence of IWR-1-endo and LY333531. The percentage of TRA-1-60 positive cells is shown in the right corner. (F) Quantified data for TRA-1-60 positive ratio (%). The percentage of TRA-1-60 positive cells is shown as mean $\pm$ SE (n=3).

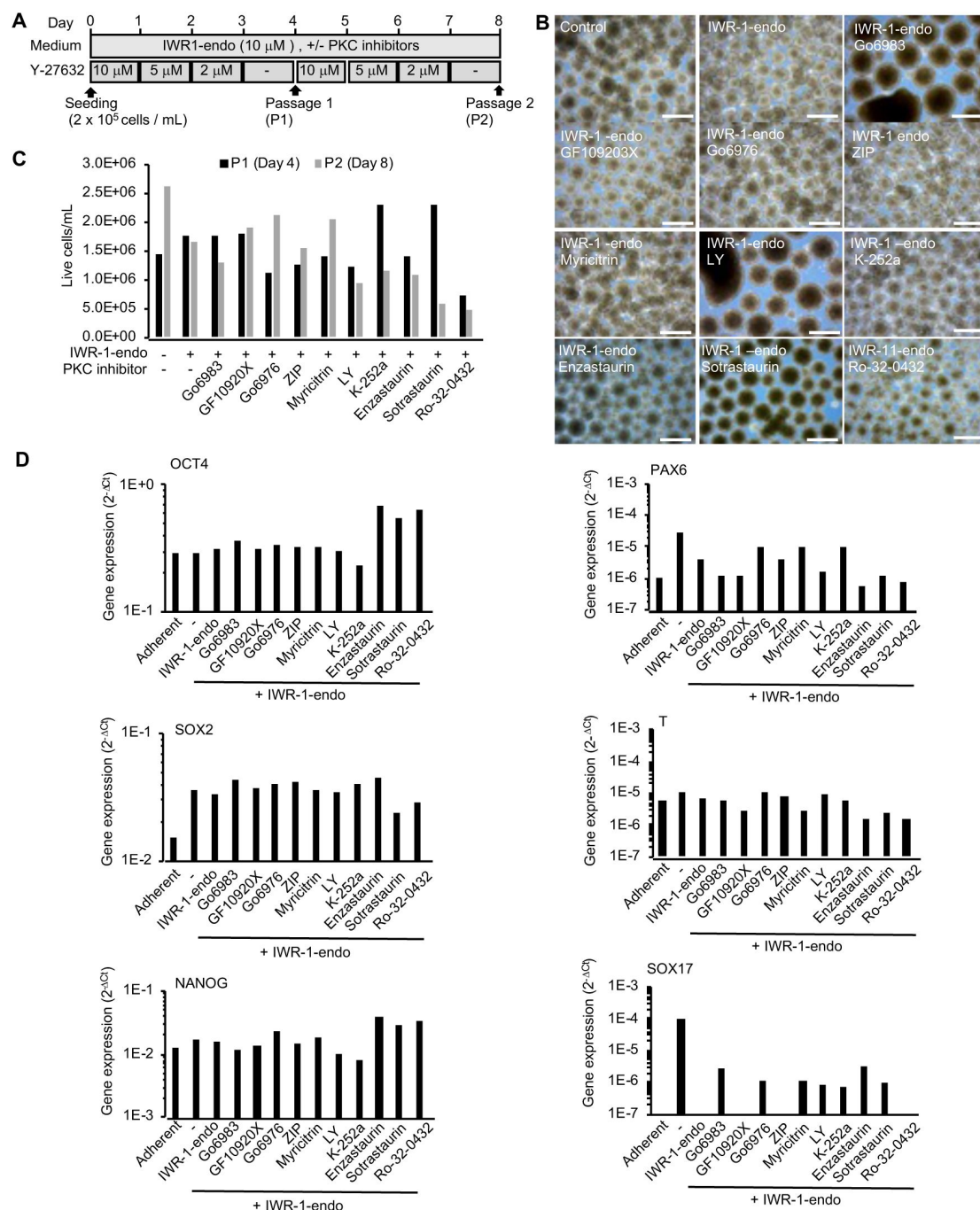


Figure 2—figure supplement 2.

Activity of different type of PKC inhibitors on spontaneous differentiation of suspension-cultured hiPSCs.

(A) Schematic representing suspension culture of hiPSCs supplemented with PKC inhibitors. Healthy donor-derived hiPSCs (201B7 line) were grown under continuous agitation in the presence of Y-27632, IWR-1-endo and PKC inhibitors. Passage was performed every four days. (B) Phase-contrast images of suspension cultured hiPSCs on day 8 (P2) in the absence or presence of IWR-1-endo and each PKC inhibitors. Scale bars, 500 μ m. (C) Cell growth of hiPSCs (201B7). Graph shows live cell numbers counted on day 4 and day 8 in suspension-culture ($n=1$). The PKC inhibitors added are labelled in the graphs. (D) Gene expression in hiPSCs cultured in adherent or suspension conditions with each PKC inhibitor. RT-qPCR was performed on cDNAs prepared from day 8 (passage 2) samples ($n=1$). qPCR was performed for pluripotency (OCT4, SOX2, NANOG) and early differentiation markers (PAX6, T, SOX17). Gene expression was normalized to beta-ACTIN.

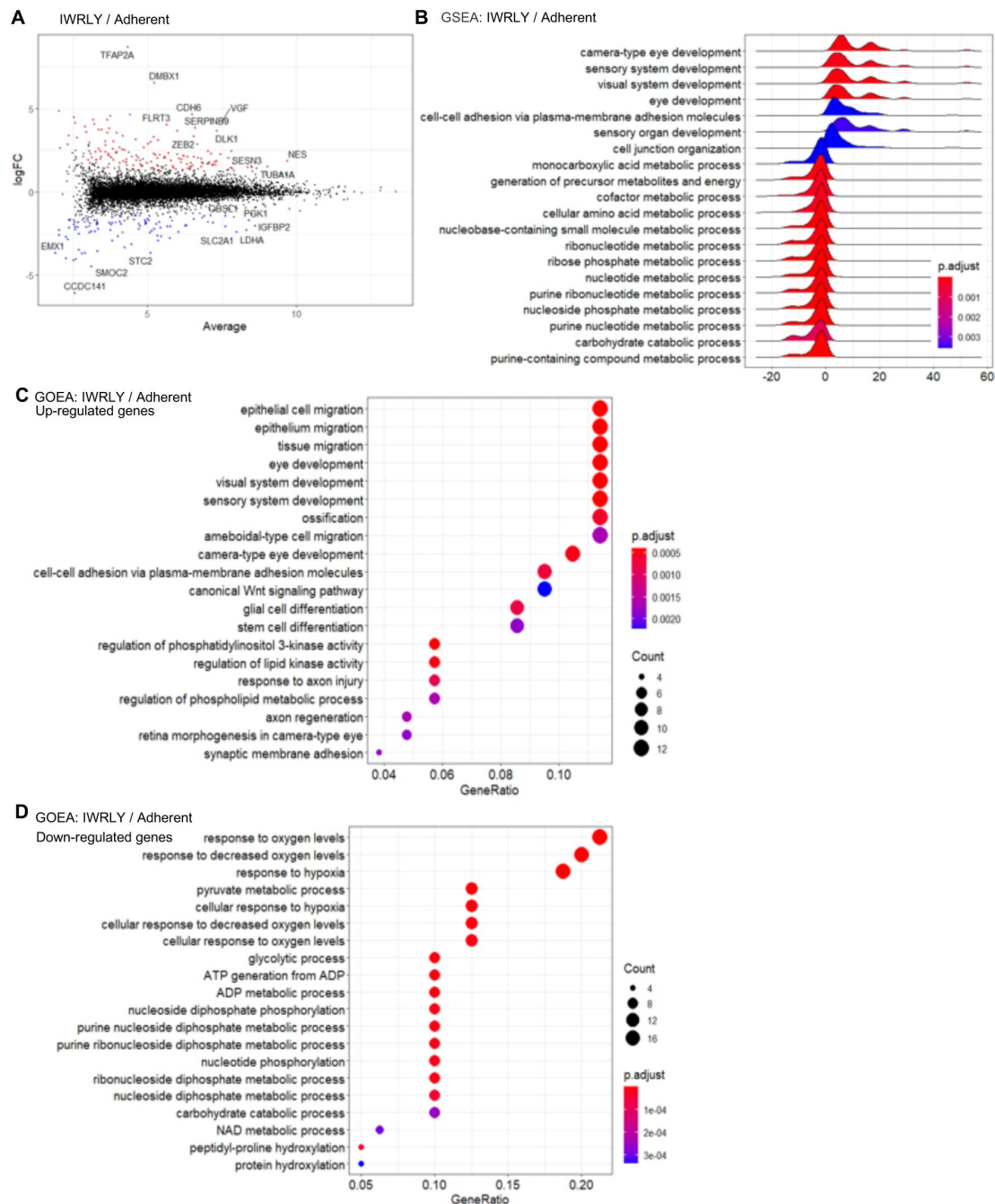


Figure 3—figure supplement 1.

Global gene expression of suspension-cultured hiPSCs with IWR-1-endo and LY333531 in comparison to adherent-cultured hiPSCs.

(A) MA plot (log2 fold change versus mean average expression) comparing the transcriptomes between adherent- and suspension-cultured hiPSCs treated with IWR-1-endo and LY333531 (IWRLY). Transcripts with log2 fold change ≥ 2 or ≤ -2 (FDR < 0.01) are highlighted with red or blue dots, respectively. The names of the representative genes are shown in the plot. (B) GSEA of the gene sets of suspension-cultured hiPSCs with IWRLY compared to adherent-cultured hiPSCs. Analysis was performed on both up-regulated and down-regulated genes. Statistically significant enrichment is shown. Adjusted P-values are illustrated from blue to red as low to high. (C and D) GOEA of the gene sets of suspension-cultured hiPSCs with IWRLY compared to adherent-cultured hiPSCs. Analysis was performed on up-regulated genes in (C) and down-regulated genes in (D), respectively. Results are ranked by significance (adjusted P values) and/or counted gene numbers.

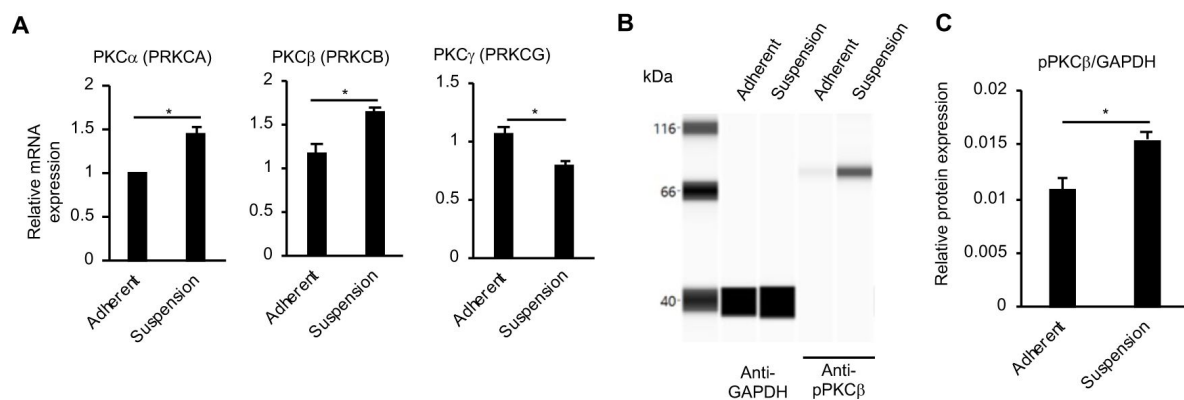


Figure 3—figure supplement 2.

Up-regulation of PKC genes in suspension-cultured hiPSCs.

(A) RNA expression of conventional PKC isoform genes in hiPSCs as detected by RT-qPCR. cDNA was prepared from adherent- or suspension-cultured hiPSCs on day 5. The gene expression was normalized to GAPDH. Results are displayed as relative fold increase to adherent-culture. Data are presented as mean $\pm$ SE (n=3). P-values were statistically analyzed by Student's t-test. (B) Detection of phosphorylated PKC β protein (pPKC β) in suspension cultured hiPSCs on day 5 using an automated capillary western blot (Simple Western) assay. Adherent-cultured hiPSCs were used as a control. (C) Quantification of pPKC β expression. The protein expression was normalized to GAPDH. Data are shown as mean $\pm$ SE (n=3). P-value was statistically analyzed using Student's t-test. Scale bars, 200 μ m.

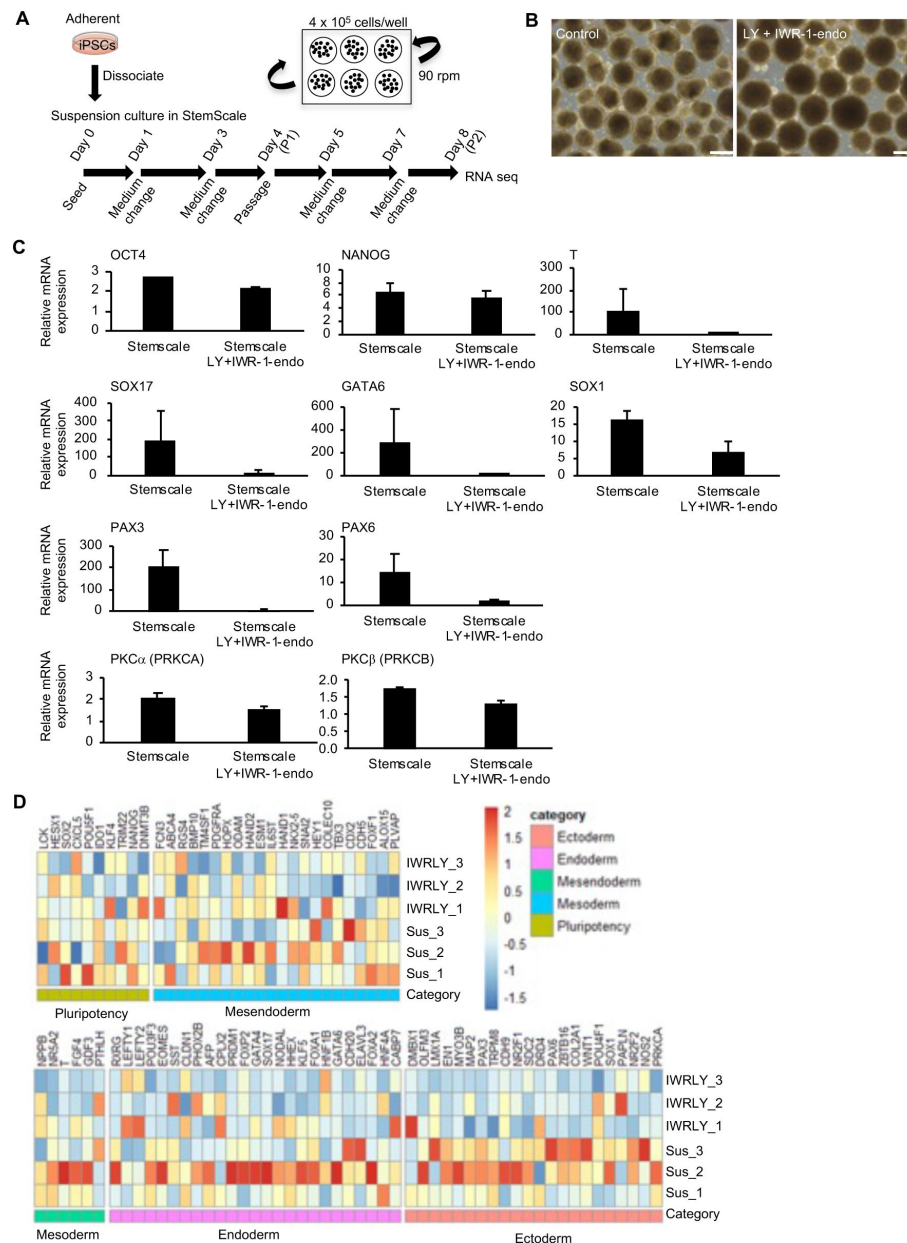


Figure 4—figure supplement 1.

Generality of the inhibitory effects of IWR-1-endo and LY333531 on spontaneous differentiation of hiPSCs cultured in suspension condition.

(A) Schematic representing suspension culture of hiPSCs using StemScale medium (Thermo Fisher Scientific) as the basic culture medium. hiPSCs were grown under continuous agitation (90 rpm) in the presence or absence of IWR-1-endo and LY333531. Passage was performed every four days according to the manufacture's protocol. cDNAs were prepared from day 8 (P2) and used for RNA seq. (B) Phase-contrast images of suspension cultured hiPSCs on day 8 in the presence or absence of IWR-1-endo and LY333531. Scale bars, 400 μ m. (C) RNA expression of pluripotency and early differentiated genes in hiPSCs, as detected by RT-qPCR. cDNA was prepared from suspension-cultured hiPSCs on day 8 in the presence or absence of IWR-1-endo and LY333531. The gene expression was normalized to GAPDH. Data are presented as mean $\pm$ SE (n=3). (D) Heat map of marker genes for pluripotency and differentiation to three germ layers from RNA-seq data in suspension-cultured iPSCs without compounds (Sus_1, Sus_2 and Sus_3) or with IWR-1-endo and LY333531 (IWRLY_1, IWRLY_2 and IWRLY_3). The listed genes were classified as pluripotent, mesendoderm, mesoderm, endoderm, and ectoderm, as defined in the Scorecard Assay (Thermo Fisher Scientific).

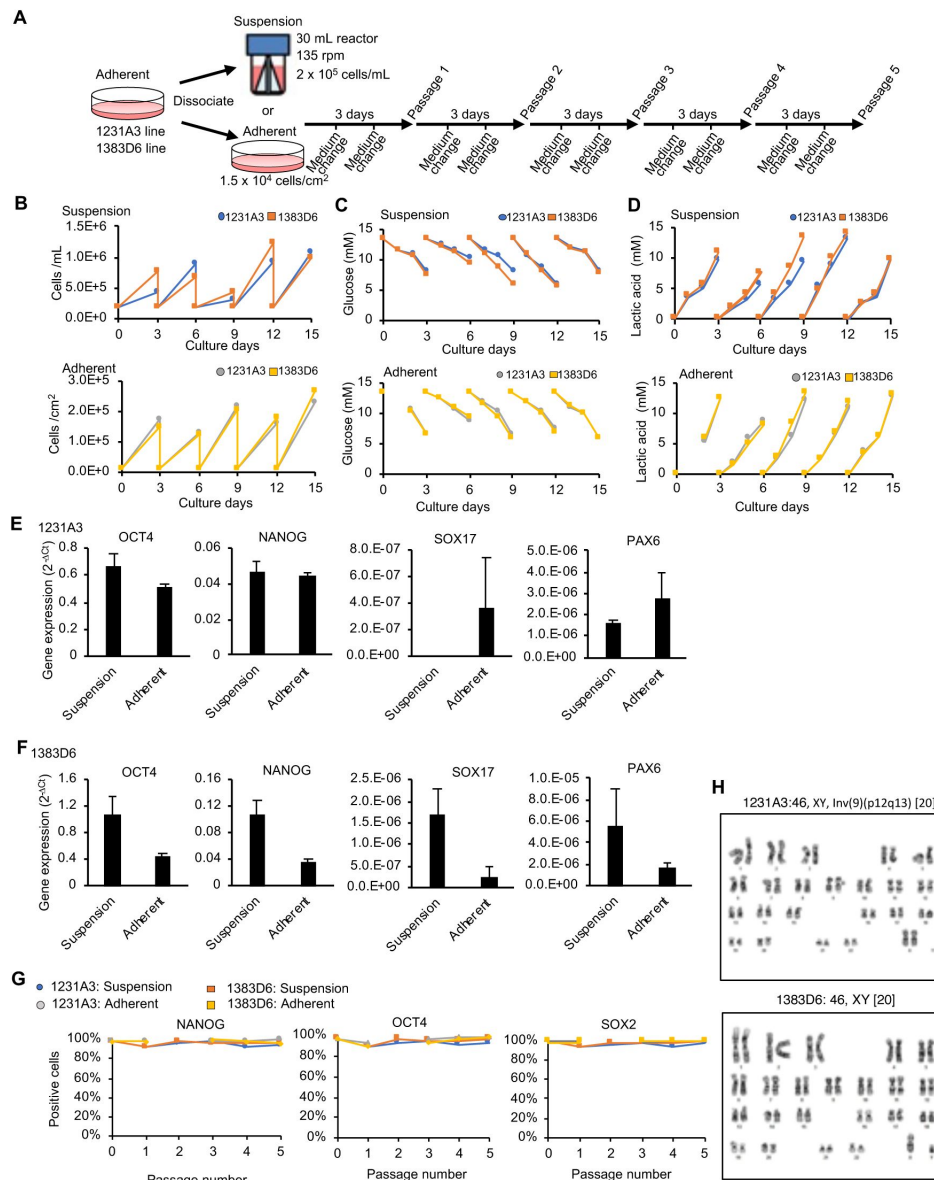


Figure 5—figure supplement 1.

Suspension-culture of hiPSCs supplemented with IWR-1-endo and LY333531 using bioreactors with continuous agitation.

(A) Schematic representing suspension culture of hiPSCs in a 30 mL bioreactor. Healthy donor-derived hiPSCs (1231A3 and 1383D6 lines) were grown with continuous agitation in the presence or absence of IWR-1-endo and LY333531. Passages were performed every three days. (B) Graphs showing cell numbers at each passage (n=1). Upper: suspension-culture of 1231A3 and 1383D6 lines. Lower: adherent-culture of 1231A3 and 1383D6 lines. (C) Graphs showing glucose concentration in the culture medium at each culture day. Upper: suspension-culture of 1231A3 and 1383D6 lines. Lower: adherent-culture of 1231A3 and 1383D6 lines. (D) Graphs showing lactic acid concentration in the culture medium at each culture day. Upper: suspension-culture of 1231A3 and 1383D6 lines. Lower: adherent-culture of 1231A3 and 1383D6 lines. (E and F) RT-qPCR analysis of hiPSCs cultured in 30 mL bioreactor or adherent conditions. cDNA samples were prepared from passage 3 and 5 of the 1231A3 in (E) and 1383D6 in (F). qPCR was performed for *OCT4* and *NANOG* (pluripotent), *CDX2* (mesoderm), *SOX17* (endoderm) and *PAX6* (neuroectoderm). The gene expression was normalized to β -ACTIN. Bar graph showing the average gene expressions of passage 3 and 5 (mean $\pm$ SE). (G) Flow cytometric analysis of suspension- or adherent-cultured 1231A3 and 1383D6 lines. Positive cells (%) of pluripotency marker proteins: NANOG, OCT4, and SOX2 are shown. (H) Karyotyping of hiPSCs cultured in 30 mL bioreactor. Cell samples were prepared on passage 5, and used for G-band karyotyping analysis. A short deletion on chromosome 9 of 1231A3 (indicated with arrow) was derived from the original line.

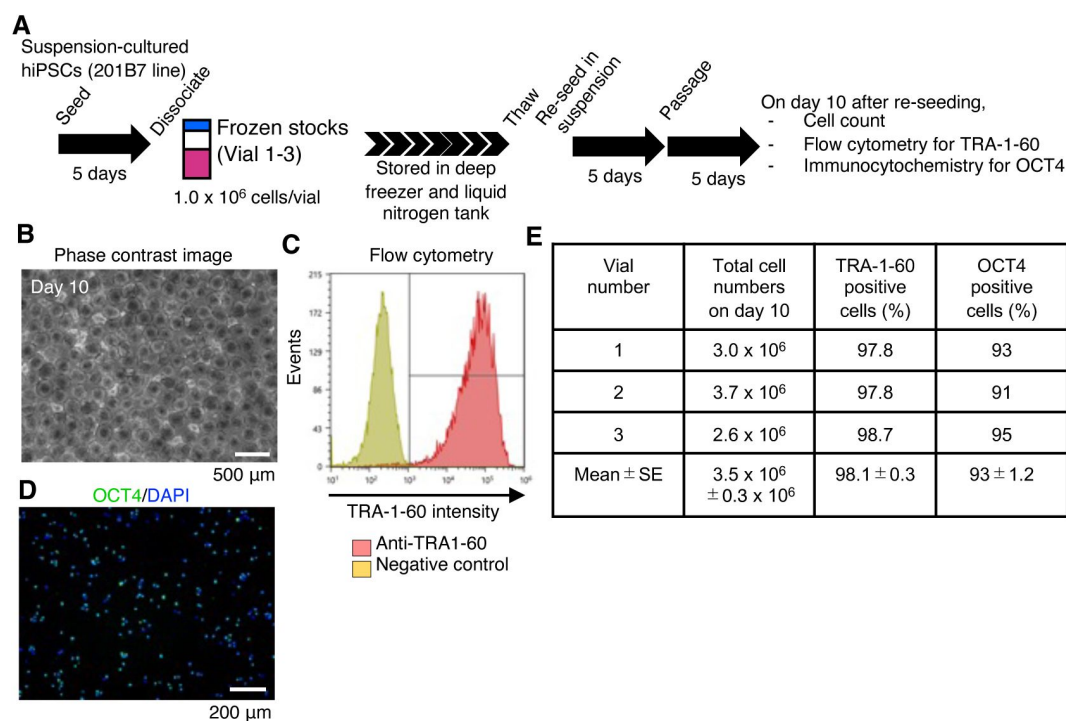


Figure 6—figure supplement 1.

Re-suspension from frozen stocks of hiPSCs under suspension culture conditions supplemented with IWR-1-endo and LY333531.

(A) Schematics of re-suspension culture of frozen stocks of single-cells-derived 201B7 clone (Vial 1-3). (B) Represented phase-contrast images on day 10 (Vial 3). Scale bars: 500 μ m. (C) Represented flow cytometry of TRA-1-60 (Vial 3). (D) Represented immunocytochemistry of OCT4 (Vial 3). Scale bars: 200 μ m. (E) Summary of the characterization of re-suspension-cultured iPSCs from frozen stocks. Total cell numbers on day 10, the ratio of TRA-1-60-positive cells (%), and the ratio of OCT4-positive cells (%) are shown (mean $\pm$ SE) (n=3).

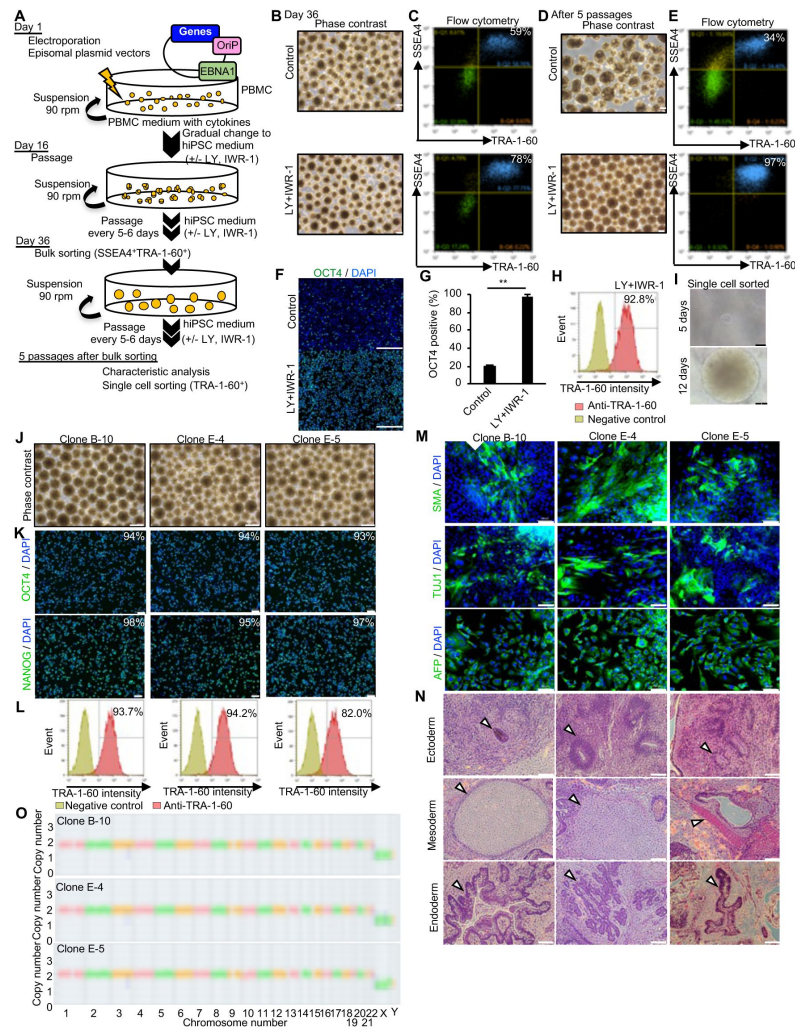


Figure 7—figure supplement 1.

Establishment of hiPSCs in completed suspension conditions using episomal vectors.

(A) Flow chart representing the establishment of hiPSCs from PBMCs by electroporation of episomal vectors. (B) Phase-contrast images of transfected cells on day 36 after electroporation of episomal vectors in the absence or presence of IWR-1-endo and LY333531. Scale bars: 400 μ m. (C) Flow cytometry of TRA-1-60 and SSEA4. TRA-1-60 and SSEA4-positive cells were sorted as the bulk fraction and further suspended in the absence or presence of IWR-1-endo and LY333531. The percentages of TRA-1-60 and SSEA4-positive cells are shown in the right corner. (D) Phase-contrast images of suspension-cultured cell aggregates after bulk sorting on passage 5. Scale bars: 400 μ m. (E) Flow cytometry of TRA-1-60 and SSEA4. The percentage of TRA-1-60- and SSEA4-positive cells are shown in the right corner. (F) Immunocytochemistry of OCT4 on sorted-cells cultured in the absence (top) or presence (bottom) of IWR-1-endo and LY333531 for 5 passages. Scale bars: 400 μ m. (G) Quantified data for OCT4-positive cell percentage. Bar graph indicating average OCT4-positive cell percentages (mean $\pm$ SE) (n=3). P-value was statistically analyzed by Student's t-test. (H) Single-cell sorting and cloning of TRA-1-60 positive cells. Flow cytometry was performed for cell fraction that was cultured in the presence of IWR-1-endo and LY333531. TRA1-60-positive cells were collected as single cells in a 96-well plate and expanded in the culture medium supplemented with Y-27632, IWR-1-endo and LY333531. (I) Representative phase-contrast images of single-sorted cell aggregates on day 5 and 12. Scale bars, 400 μ m. (J) Phase-contrast images of established clones (B-10, E-4, and E-5). Scale bars: 400 μ m. (K) Immunocytochemistry of OCT4 and NANOG in these clones. The percentage of positive cell are shown. Scale bars: 100 μ m. (L) Flow cytometry of TRA-1-60 in these clones. TRA-1-60 positive percentages are shown in the right corner. (M) Immunocytochemistry of differentiated cells in EBs from these clones. Anti-TUJ1, -SMA and -AFP antibodies were used. Scale bars: 100 μ m. (N) Hematoxylin-Eosin staining of paraffin-embedded sections of teratoma derived from these clones. White arrowheads indicate representative tissue structures of the ectoderm, mesoderm, or endoderm. Scale bars, 100 μ m. (O) Chromosomal copy numbers detected by CGH array analysis (Karyostat assay) of these clones.

Name	Effect	Manufacturer	Identifier	Concentration
CHIR99021	Wnt signal activator	Fujifilm Wako Pure Chemical	034-23103	3 μ M
IWP-2	Wnt signaling inhibitor	Fujifilm Wako Pure Chemical	034-24301	5 μ M
IWR-1-endo	Wnt signaling inhibitor	Fujifilm Wako Pure Chemical	037-25131	10 μ M
Go6976	PKC (α , β) inhibitor	Fujifilm Wako Pure Chemical	071-06551	5 μ M
Go6983	PKC (α , β , γ , δ) inhibitor	Fujifilm Wako Pure Chemical	078-06443	5 μ M
GF109203X	PKC (α , β , γ) inhibitor	Tocris Bioscience	0741	5 μ M
Enzastaurin	PKC (α , β , γ , ϵ) inhibitor	Sigma-Aldrich	SML0762	5 μ M
K-252a	PKC (μ) inhibitor	Sigma-Aldrich	K1639	5 μ M
LY333531 (Ruboxistaurin)	PKC (β) inhibitor	Sigma-Aldrich	SML0693	5 μ M
Myricitrin	PKC (α , ϵ) inhibitor	Sigma-Aldrich	91255	5 μ M
Sotrastaurin	PKC (α , β , δ , ϵ , η , θ) inhibitor	Cayman	16726	5 μ M
ZIP	PKC (ζ) inhibitor	Tocris Bioscience	2549	5 μ M
Ro-32-0432	PKC (α , β , γ , ϵ) inhibitor	Tocris Bioscience	1587	5 μ M
FK506	Calcineurin inhibitor	Fujifilm Wako Pure Chemical	063-06071	100 ng/mL
Cyclosporine A (CsA)	Calcineurin inhibitor	Fujifilm Wako Pure Chemical	031-24931	1 mg/mL
DAPT	γ -secretase inhibitor	Fujifilm Wako Pure Chemical	043-33581	10 μ M
BMP4 (Human, recombinant)	BMP-SMAD signaling activator	Fujifilm Wako Pure Chemical	020-18851	40 ng/mL
LIF (Human, recombinant)	LIF-STAT signaling activator	Nacalai Tesque	NU0013-1	2000 units/mL
Valproic acid (VPA)	Epigenetic modifier	Fujifilm Wako Pure Chemical	227-01071	0.5 mM
Ascorbic acid	Epigenetic modifier	Nacalai Tesque	13048-42	50 ng/mL

Supplementary Table 1.

The list of chemicals and cytokines used in the screening assay.

Target gene	Taqman Assay ID or sequence
ACTB	F: cctcatgaagatcctcaccga R: ttgccaatgggtgatgacctgg
CDX2	F: caccacagccatagacctac R: gtcagtccaggcaatgcttc
CXCR4	F: actgagaagcatgacggacaag R: aggtagcgggtccagactgatg
GAPDH	Hs02786624_g1
GATA6	Hs00232018_m1
NANOG	Hs02387400_g1
OCT4 (POU5F1)	Hs04260367_gH
PAX3	Hs00240950_m1
PAX6	Hs01088114_m1
PDGFR α	F: gctgagcctaactcctctgcc R: actgctcactccaagaccg
PKC α (PRKCA)	Hs00925200_m1
PKC β (PRKCB)	Hs00176998_m1
PKC γ (PRKCG)	Hs00177010_m1
SOX1	Hs01057642_s1
SOX2	F: caccaatcccatccactcac R: gcaaagctcctaccgtaccac
SOX17	Hs00751752_s1
T	Hs00610080_m1
SeV detection	F: accaacaggcgggtggtgcaa R: tccacccaaccctagcgt
PAX6 genotyping	F: ggaattccagtacttcacgtgaaggcatct R: tagaactgaagcggcttaacagccatttt
SOX17 genotyping	F: agaaagggtgccttagaggacgggtgtt R: aggaagtgtgtaacactgcttctggcctgc

Supplementary Table 2.

The list of Taqman probes and primer sets used in this study.

Name	Host species	Dilutions or Concentrations	SOURCE	IDENTIFIER
Alpha-Fetoprotein (AFP)	Mouse	1 μ g/mL	R&D systems	Cat#MAB1368
Alpha-SMA	Mouse	1 μ g/mL	R&D systems	Cat#MAB1420
Cardiac Troponin T	Mouse	10 μ g/mL	Thermo Fisher Scientific	Cat#MA5-12960
CORIN	Mouse	10 μ g/mL	Sigma-Aldrich	Cat#WH0010699M1
GAPDH	Mouse	1 μ g/mL	R&D systems	Cat#MAB5718
NANOG	Rabbit	0.5 μ g/mL	Reprocell	Cat#RCAB004P-F
OCT-3/4	Mouse	0.4 μ g/mL	Santa Cruz	Cat#sc-5279
Oct-4A (C30A3) Rabbit mAb (Alexa Fluor 488 Conjugate)	Rabbit	1:30	Cell Signaling Technology	Cat# 5177S
PAX6	Rabbit	1:400 (Immunocytochemistry)	MBL	Cat#PD022
PAX6	Sheep	5 μ g/mL (Wes)	R&D Systems	Cat#AF8150
Anti-PKC beta1 + PKC beta2 (phospho T641)	Rabbit	1:50	Abcam	Cat#ab194749
SOX17	Goat	0.4 μ g/mL (Immunocytochemistry) 1 μ g/mL (Wes)	R&D systems	Cat#AF1924
FITC Mouse anti-SSEA-4	Mouse	1:5	BD Bioscience	Cat#560126
APC anti-human SSEA4	Mouse	1:20	BioLegend	Cat#330418
Alexa Fluor 488 anti-human TRA1-60-R	Mouse	1:20	BioLegend	Cat#330614
Alexa Fluor 488 Mouse anti-Human TRA-1-60 Antigen	Mouse	1:10	BD Bioscience	Cat#560173
TUJ1	Mouse	1 μ g/mL	R&D systems	Cat#MAB1195

Supplementary Table 3.

The list of primary antibodies used in this study.

Name	Host species	Dilutions	SOURCE	IDENTIFIER
Anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488	Donkey	1:1000	Thermo Fisher Scientific	Cat#A-21202
DyLight 488 anti-rabbit IgG (minimal x-reactivity)	Donkey	1:1000	BioLegend	Cat#406404
Anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555	Donkey	1:1000	Thermo Fisher Scientific	Cat#A-31570
Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Goat	1:1000	Thermo Fisher Scientific	Cat#A-21428
Anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Goat	1:1000	Thermo Fisher Scientific	Cat#A-11001
Donkey Anti-Sheep IgG HRP Affinity Purified PAb	Sheep	1:50	R&D systems	Cat# HAF016

Supplementary Table 4.

The list of secondary antibodies used in this study.

Name	Source	URL
clusterProfiler (v3.16.1)	R package	https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
edgeR (v3.30.3)	R package	https://bioconductor.org/packages/release/bioc/html/edgeR.html
R (version4.1.0)	R package	https://www.r-project.org/
CLC Genomics Workbench (v20)	QIAGEN	https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/
Cell Sorter software	Sony	https://www.sonybiotechnology.com/us/instruments/sh800s-cell-sorter/software/
Compass software for Simple Western	Protein Simple	https://www.biotechne.com/resources/instrument-software-download-center/compass-software-simple-western
BZ-X800 microscope analysis software	Keyence	https://www.keyence.com/landing/microscope/lp_fluorescence.jsp
QuantStudio 3 and 5 Real-Time PCR System Software	Thermo Fisher Scientific	https://www.thermofisher.com/jp/ja/home/global/forms/life-science/quantstudio-3-5-software.html
Chromosome Analysis Suite (ChAS) and Affymetrix GeneChip Command Console software programs	Thermo Fisher Scientific	https://www.thermofisher.com/jp/ja/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/chromosome-analysis-suite.html
CellSens microscope software	Evident (Olympus)	https://www.olympus-lifescience.com/en/support/downloads/

Supplementary Table 5.

Software used in this study.

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Editors

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Reviewer #1 (Public Review):

Summary:

The authors have presented data showing that there is a greater amount of spontaneous differentiation in human pluripotent cells cultured in suspension vs static and have used PKC β and Wnt signaling pathway inhibitors to decrease the amount of differentiation in suspension culture.

Strengths:

This is a very comprehensive study that uses a number of different reactor designs and scales in addition to a number of unbiased outcomes to determine how suspension impacts the behaviour of the cells and in turn how the addition of inhibitors counteracts this effect. Furthermore, the authors were also able to derive new hiPSC lines in suspension with this adapted protocol.

Weaknesses:

The main weakness of this study is the lack of optimization with each bioreactor change. It has been shown multiple times in the literature that the expansion and behaviour of pluripotent cells can be dramatically impacted by impeller shape, RPM, reactor design, and multiple other factors. It remains unclear to me how much of the results the authors observed (e.g. increased spontaneous differentiation) was due to not having an optimized bioreactor protocol in place (per bioreactor vessel type). For instance - was the starting seeding density, RPM, impeller shape, feeding schedule, and/or any other aspect optimized for any of the reactors used in the study, and if not, how were the values used in the study determined?

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Reviewer #2 (Public Review):

This study by Matsuo-Takasaki et al. reported the development of a novel suspension culture system for hiPSC maintenance using Wnt/PKC inhibitors. The authors showed elegantly that inhibition of the Wnt and PKC signaling pathways would repress spontaneous differentiation into neuroectoderm and mesendoderm in hiPSCs, thereby maintaining cell pluripotency in suspension culture. This is a solid study with substantial data to demonstrate the quality of the hiPSC maintained in the suspension culture system, including long-term maintenance in >10 passages, robust effect in multiple hiPSC lines, and a panel of conventional hiPSC QC assays. Notably, large-scale expansion of a clinical grade hiPSC using a bioreactor was also demonstrated, which highlighted the translational value of the findings here. In addition, the author demonstrated a wide range of applications for the IWR1+LY suspension culture system, including support for freezing/thawing and PBMC-iPSC generation in suspension culture format. The novel suspension culture system reported here is exciting, with significant implications in simplifying the current culture method of iPSC and upscaling iPSC manufacturing.

Another potential advantage that perhaps wasn't well discussed in the manuscript is the reported suspension culture system does not require additional ECM to provide biophysical support for iPSC, which differentiates from previous studies using hydrogel and this should further simplify the hiPSC culture protocol.

Interestingly, although several hiPSC suspension media are currently available commercially, the content of these suspension media remained proprietary, as such the signaling that represses differentiation/maintains pluripotency in hiPSC suspension culture remained unclear. This study provided clear evidence that inhibition of the Wnt/PKC pathways is critical to repress spontaneous differentiation in hiPSC suspension culture.

I have several concerns that the authors should address, in particular, it is important to benchmark the reported suspension system with the current conventional culture system (eg adherent feeder-free culture), which will be important to evaluate the usefulness of the reported suspension system. Also, the manuscript lacks a clear description of a consistent robust effect in hiPSC maintenance across multiple cell lines. There are also several minor comments that should be addressed to improve readability, including some modifications to the wording to better reflect the results and conclusions.

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Reviewer #3 (Public Review):

In the current manuscript, Matsuo-Takasaki et al. have demonstrated that the addition of PKC β and WNT signaling pathway inhibitors to the suspension cultures of iPSCs suppresses spontaneous differentiation. These conditions are suitable for large-scale expansion of iPSCs. The authors have shown that they can perform single-cell cloning, direct cryopreservation, and iPSC derivation from PBMCs in these conditions. Moreover, the authors have performed a thorough characterization of iPSCs cultured in these conditions, including an assessment of undifferentiated stem cell markers and genetic stability. The authors have elegantly shown that iPSCs cultured in these conditions can be differentiated into derivatives of three germ layers. By differentiating iPSCs into dopaminergic neural progenitors, cardiomyocytes, and hepatocytes they have shown that differentiation is comparable to adherent cultures. This new method of expanding iPSCs will benefit the clinical applications of iPSCs.

Recently, multiple protocols have been optimized for culturing human pluripotent stem cells in suspension conditions and their expansion. Additionally, a variety of commercially available media for suspension cultures are also accessible. However, the authors have not

adequately justified why their conditions are superior to previously published protocols (indicated in Table 1) and commercially available media. They have not conducted direct comparisons. Additionally, the authors have not adequately addressed the observed variability among iPSC lines. While they claim in the Materials and Methods section to have tested multiple pluripotent stem cell lines, they do not clarify in the Results section which line they used for specific experiments and the rationale behind their choices. There is a lack of comparison among the different cell lines. It would also be beneficial to include testing with human embryonic stem cell lines. Additionally, there is a lack of information regarding the specific role of the two small molecules in these conditions. The authors have not attempted to elucidate the underlying mechanism other than RNA expression analysis.

For these reasons some aspects of the manuscript need to be extended:

1. It is crucial for authors to specify the culture media used for suspension cultures. In the Materials and Methods section, the authors mentioned that cells in suspension were cultured in either StemFit AK02N medium, 415 StemFit AK03N (Cat# AK03N, Ajinomoto, Co., Ltd., Tokyo, Japan), or StemScale PSC416 suspension medium (A4965001, Thermo Fisher Scientific, MA, USA). The authors should clarify in the text which medium was used for suspension cultures and whether they observed any differences among these media.
2. In the Materials and Methods section, the authors mentioned that they used multiple cell lines for this study. However, it is not clear in the text which cell lines were used for various experiments. Since there is considerable variation among iPSC lines, I suggest that the authors simultaneously compare 2 to 3 pluripotent stem cell lines for expansion, differentiation, etc.
3. Single-cell sorting can be confusing. Can iPSCs grown in suspensions be single-cell sorted? Additionally, what was the cloning efficiency? The cloning efficiency should be compared with adherent cultures.
4. The authors have not addressed the naïve pluripotent state in their suspension cultures, even though PKC inhibition has been shown to drive cells toward this state. I suggest the authors measure the expression of a few naïve pluripotent state markers and compare them with adherent cultures

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