A stress-reduced passaging technique improves the viability of human pluripotent cells

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Highlights
- An improved passaging method significantly increases the viability of human PSCs
- The method triggers less DNA damage and apoptosis compared to the conventional method
- The stress-reduced method improves the results of downstream applications
- The method attenuates the overgrowth of highly viable abnormal subpopulations

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In brief
Takahashi et al. modify the passaging method for human pluripotent stem cells cultured on recombinant matrices. The revised protocol increases cell viability by reducing DNA damage and apoptosis, improves the efficiency and reproducibility of downstream applications, and attenuates the overgrowth of abnormal cell populations.
MOTIVATION
Although chemically defined culture systems for human pluripotent stem cells (PSCs) are in widespread use, cell viability after passaging among PSC clones is often variable, leading to issues with reproducibility. To improve the efficiency and reproducibility of advanced PSC culture systems, we have developed an improved passaging method by optimizing procedures for cell detachment and dissociation. The revised passaging method improves cell viability and makes downstream applications more efficient and reproducible.

SUMMARY
Xeno-free culture systems have expanded the clinical and industrial application of human pluripotent stem cells (PSCs). However, reproducibility issues, often arising from variability during passaging steps, remain. Here, we describe an improved method for the subculture of human PSCs. The revised method significantly enhances the viability of human PSCs by lowering DNA damage and apoptosis, resulting in more efficient and reproducible downstream applications such as gene editing and directed differentiation. Furthermore, the method does not alter PSC characteristics after long-term culture and attenuates the growth advantage of abnormal subpopulations. This robust passaging method minimizes experimental error and reduces the rate of PSCs failing quality control of human PSC research and application.

INTRODUCTION
Xeno-free culture methods for human pluripotent stem cells (PSCs) are widespread. They allow the maintenance of human PSCs, such as embryonic stem cells (ESCs) and induced PSCs (iPSCs), without losing their differentiation potential. The development and optimization of culture vessel-coating reagents, such as synthetic substrates (Melkoumian et al., 2010; Villa-Diaz et al., 2010) and extracellular matrices (Rodin et al., 2010), have contributed to efficient culture systems without the inclusion of animal-derived components, including feeder cells and the Engelbreth-Holm-Swarm mouse tumor-derived basement membrane matrix (i.e., Matrigel) (Thomson et al., 1998; Xu et al., 2001). In particular, recombinant extracellular matrices, such as laminin and vitronectin, allow the rapid and efficient adhesion of undifferentiated PSCs, which express compatible integrin receptors (Chen et al., 2011; Miyazaki et al., 2012; Nakagawa et al., 2014; Rodin et al., 2010; Xu et al., 2001).

Combining such fine-tuned culture systems with inhibitors of cell death, such as the Rho-associated protein kinase (ROCK) inhibitor, dramatically improves the survival of human PSCs during passage and makes the single-cell culture of human PSCs possible (Chen et al., 2021; Watanabe et al., 2007). Furthermore, single-cell cloning following gene disruption or correction by CRISPR/Cas9 facilitates the isolation of PSCs for in vitro disease modeling and as possible sources for cell therapies (Hotta and Yamanaka, 2015). In this way, the single-cell culture of human PSCs is a promising method for the systematic quality control of clinical-grade cell lines and reproducibility of basic research.

Unlike advances in the extracellular environment, the passaging procedure has not changed significantly in the last decade. Conventional passaging methods for human PSCs have adhered fundamentally to the classical procedure of dissociating cells after removing/neutralizing the enzymatic activity and/or a chelating reaction by adding growth media (Beers et al., 2012; Chen et al., 2011; Miyazaki et al., 2012; Nakagawa et al., 2014). Considering that the PSC culture conditions have changed significantly, there is room for optimizing the passaging procedure to fit current culture protocols. This study revised a passaging protocol for human PSCs cultured in xeno-free
conditions. The method significantly improves the reproducibility of experiments and cell viability. Such controllable passaging facilitated the results of several downstream assays, such as gene editing and differentiation. The modification of the passaging procedure did not change the PSC characteristics, but it did attenuate the growth advantage of abnormal subpopulations. The revised method promises to make PSC cultures safer and further expands the range of human PSC usage.

RESULTS

The conventional method of passaging human PSCs on a xeno-free culture described previously (Figure 1A) (Nakagawa et al., 2014) results in poor reproducibility such as a wide range of cell viability (Figure 1B). To resolve this issue, we modified the procedure of cell passaging and explored the effects on cell viability in detail using two independent iPSC lines (Table S1), WTB6 (Miyakawa et al., 2014) and WTC11 (Kreitzer et al., 2013), maintained on laminin-511 E8 fragment in a chemically defined media, StemFit (Miyazaki et al., 2012; Nakagawa et al., 2014) (Figure 1A). First, we used TrypLE, 5 mM EDTA, or AccuMax (Kim et al., 2016) as the detachment reagent instead of a 1:1 mix of TrypLE and 0.5 mM EDTA (hereafter T1E1), which is used in the conventional method (Miyazaki et al., 2017; Nakagawa et al., 2014). Second, the incubation time was extended from 5 to 10 min. Finally, the dissociation to single cells was performed directly in the detachment solution rather than replacing the detachment solution with growth media and dislodging the cells from the culture vessel using a cell scraper before dissociation. Using this revised method, we could easily detach the cells with all three detachment reagents by gently pipetting up and down. This was nearly impossible with the conventional protocol, which required the use of a cell scraper. As a result of the modifications, the revised method significantly and reproducibly improved the viability of human PSCs (Figures 1B and S1A). In addition to WTB6 and WTC11, the method improved the viability of the iPSC lines such as 201B7 (Takahashi et al., 2007) and 585A1 (Okita et al., 2011) and the ESC line H9 (Thomson et al., 1998) (Table S1) >95% on average (Figure S1B). The revised method also worked well for another xeno-free PSC culture condition combining recombinant vitronectin and Essential 8 media (Chen et al., 2011) (Figure S1C). Since EDTA has been used for the detachment of human PSCs from laminin-511 or vitronectin-coated culture vessels, we tested 5 mM EDTA instead of TrypLE in the revised method (Beers et al., 2012; Chen et al., 2011; Miyazaki et al., 2012). After 10 min of incubation and then dissociation in 5 mM EDTA, the cells were easily detached using a micropipette and showed high viability (Figure 1B). No apparent cytotoxicity was detected in 0.5–5 mM EDTA (Figure S1D), although TrypLE but not EDTA generated single-cell suspensions efficiently by pipetting (Figure S1E). This observation suggests that we can choose TrypLE or EDTA according to the type of downstream assay. These data indicate higher viability of human PSCs in the passaging process.

Next, we systematically tested the detachment and dissociation processes to elucidate which part of the passaging is crucial for viability. We detached human PSCs using TrypLE or T1E1 and then harvested the cells in detachment solution or growth media. We found that the cell viability depended on the condition of the cell dissociation process regardless of the components of the detachment solution (Figure 1C). Using a cell scraper in detachment solution did not decrease the viability, suggesting that damage induced by scraping is not responsible for the cell death in the conventional method (Figure 1D). The data shown in Figures 1C and 1D led us to hypothesize that the enforced cell tearing after replacing the detachment reagent with growth media decreased the cell viability. After replacing the detachment solution with growth media, the cells immediately began to re-attach to the culture vessels, leading to difficult and inefficient cell detachment by pipetting (Figure S1F). Moreover, a longer incubation time in growth media before cell scraping significantly decreased cell viability (Figure 1E). However, prolonged incubation of human PSCs in TrypLE solution did not affect the viability (Figures 1E and S1G). All in all, we concluded that cell dissociation in growth media is a critical step for cell viability.

We found that the revised method not only improves the cell viability immediately after harvesting but also significantly enhances human PSCs’ adhesion to the culture vessel upon plating compared to cells collected using the conventional method if the same number of live cells are plated (Figures 1F, 1G, and S1H–S1M) (Miyazaki et al., 2012). Given that plating the same number of live cells just after harvesting by different methods made such a significant difference in the adhesion efficiency, these data suggest that further cell death after plating is a cause of compromised plating efficiency. Antibody-based quantitative protein analysis revealed that a DNA damage marker, phosphorylated Ser139 of histone H2AX variant (γH2AX), and an apoptosis marker, cleaved caspase-3, were significantly increased in cells harvested by the conventional method compared to the revised method (Figures 1I and S2). These data suggest that the revised passaging method improves the viability and adhesion efficiency of human PSCs by not inducing DNA damage or apoptosis.

Based on these results, we thought that such a robust passaging could improve the efficiency and accuracy of downstream experiments. We tested whether the revised passaging method increases the yield of clones gene edited by a CRISPR/Cas9 platform. As expected, the cells harvested using the revised method before electroporation generated higher efficiency when targeting the adeno-associated virus integration site 1 (AAVS1) locus than the conventional method (Figure 2A). Next, we examined whether plating an accurate number of cells with high viability facilitates the differentiation of PSCs. Again, as expected, the revised method yielded more troponin T positive cardiomyocytes reproducibly by directed differentiation than cells harvested by the conventional method, which instead caused massive cell death or poor differentiation (Figures 2B and 2C) (Lian et al., 2012). Moreover, the PSCs harvested by
Figure 1. The revised passaging method improves the viability of human PSCs
(A) A flow diagram of the cell passage methods used in the study.
(B) The viability of human PSCs after harvesting using the conventional (Conv.) or revised (Rev.) passaging method. *p < 0.05 versus Conv., one-way ANOVA (Tukey’s multiple comparisons test); n = 10.
(C) The viability of human PSCs after harvesting using the Conv. or Rev. passaging method with T1E1 or TrypLE. *p < 0.05 versus Conv., one-way ANOVA (Tukey’s multiple comparisons test); n = 5.
(D) The viability of human PSCs after dissociation in growth media (Conv.) or dissociation reagent (Rev.) by pipetting (P) or using a cell scraper (S). *p < 0.05 versus Conv., one-way ANOVA (Tukey’s multiple comparisons test); n = 5.
(E) The viability of human PSCs harvested after incubation in dissociation reagent or growth media for the indicated times. *p < 0.05 versus 0.5 min, one-way ANOVA (Tukey’s multiple comparisons test); n = 5.
(F) Cell death after plating. Live and dead cells were visualized with calcein (green) and propidium iodide (PI, red), respectively. Bars, 100 μm.
(G) Quantification of dead cells after the indicated times of plating. Ten microscopic views were randomly imaged in each condition and time point, and the number of PI-stained dead cells were counted. *p < 0.05 versus Conv., unpaired t test; n = 10.
(H) The number of colonies derived from PSCs harvested using the Conv. or Rev. method. The mandarin-colored broken line indicates 100% adhesion efficiency. *p < 0.05 versus Conv., unpaired t test; n = 5.
(I) Quantification of DNA damage and cell death marker protein expression. Shown are the virtual blots of phospho-H2AX (γH2AX), cleaved caspase-3 (active CASP3), caspase-3 (CASP3), and VINCULIN in WTB6 iPSCs harvested using the Conv. or Rev. method.
the revised method efficiently formed neurospheres with few dead cells (Figure 2D) and could produce larger and more uniform-sized spheres than the cells harvested by the conventional method (Figure 2E). These data suggest that the revised passaging method makes downstream applications using human PSCs more efficient and controllable.

Because the revised method dissociates and collects the cells in the detachment reagent, we spun the cells down to remove the supernatant and resuspended the cell pellet in growth media (Figure 1A). The cells suspended in media containing 10% volume of dissociation reagent displayed significantly less adhesion after 18 h of plating, suggesting that removing the detachment reagent is needed (Figures 2F and 2G). Considering our intention to apply the method to automated and/or high-throughput cell culture systems for industrial applications and clinical use, excluding the centrifuge process would be more practical. Therefore, taking advantage of the characteristic that human PSCs rapidly adhere to the culture vessel in the presence of a suitable extracellular matrix, such as laminin-511, we tested whether media containing 10% of the detachment reagent can

Figure 2. The revised passaging method improves the downstream applications of human PSCs

(A) The yield of AAVS1-targeted colonies. Shown are the number of puromycin-resistant alkaline phosphatase-positive colonies derived from 50,000 iPSCs transfected with the AAVS1 targeting vector and Cas9/sgRNA expression vector. Precisely targeted clones are selected in the presence of puromycin. *p < 0.05 versus Conv., unpaired t test; n = 5.

(B) Cardiac differentiation. Representative images of cardiac troponin-T staining (cTNT, green) derived from WTB6 iPSCs are shown. Nuclei were visualized with Hoechst 33342 (blue). Bars, 100 μm.

(C) The quantitative expression analyses of marker gene expression. The heatmap shows relative expressions of the PSC and cardiomyocyte (CM) markers in undifferentiated iPSC, 3 replicates of iPSC-derived CM (PSC-CM) treated with the revised method before the differentiation induction, and fetal heart analyzed by qRT-PCR. Values are normalized by ACTB and compared to iPSC; n = 2. See also Table S2.

(D) Neurosphere formation. Representative images of phase contrast and calcein/PI-stained neurospheres derived from WTB6 iPSCs after 18 h of plating in AggreWell800. Bars, 100 μm.

(E) The diameters of spheres labeled with calcein derived from WTB6 iPSCs harvested using the Conv. (n = 116) or Rev. (n = 114) method were measured. *p < 0.05 versus Conv., unpaired t test.

(F) The effects of TrypLE on the adhesion of PSCs. WTB6 iPSCs harvested with the Rev. method were plated in media containing TrypLE. The media was changed at the indicated times after plating or not at all (no change). Microscopic views under a 10× objective were randomly chosen, and the number of nuclei labeled by Hoechst 33,342 were counted. The mandarin-colored broken line indicates 100% adhesion efficiency. *p < 0.05 versus Spin., one-way ANOVA (Tukey’s multiple comparisons test); n = 10.

(G) The effects of dissociation buffers on the adhesion of PSCs. Cells harvested with the revised method were plated in media containing a 10% volume of D-PBS, TrypLE, 5 mM EDTA, or AccuMax. Microscopic views under a 10× objective were randomly chosen, and the number of nuclei labeled by Hoechst 33342 were counted. The mandarin-colored broken line indicates 100% adhesion efficiency. *p < 0.05 versus Spin., one-way ANOVA (Tukey’s multiple comparisons test); n = 10.
Figure 3. The revised passaging method does not alter PSC characteristics

(A) Representative images of the competition between wild-type (Clover-negative) and BCL2L1-Tg (Clover-positive) cells at the indicated passage numbers. Nuclei were visualized with Hoechst 33342 (blue). Bars, 100 μm.

(B) The percentage of BCL2L1-Tg cells during the competition culture measured by genomic PCR. The mandarin-colored broken line indicates the percentage of BCL2L1-Tg cells before the competition. *p < 0.05 versus Conv., unpaired t test; n = 3.

(C) PSC markers in WTB6 iPSCs after 30 passages with the revised method. Nuclei were visualized with Hoechst 33342 (blue). Bars, 200 μm.

(D) The RNA expression. Shown are clustering analysis with heatmap of the sample-to-sample distance of global RNA expression (left) and the PSC and differentiation-defective (DD) marker expression (right) between WTB6 iPSCs before and after 30 passages with the revised method; n = 3.

(E) The protein expression. The volcano plot shows the comparison of global protein expression between WTB6 iPSCs before and after cultivation with passaging using the revised method (≥ 30 passages); n = 3. The mandarin-colored and turquoise-colored broken lines indicate p = 0.05 and fold change = 2, respectively. The red dots indicate differentially expressed proteins such as CNEP1R1, MATK, and Trypsin.

(legend continued on next page)
be replaced with complete growth media after plating. The number of attached cells after 18 h of plating decreased by half by changing the media 5 min after plating (Figure 2F). It is worth noting that changing the media 10 min after plating resulted in a 76.5% ± 14.0% yield based on the number of cells suspended in complete media before plating. The lost quarter of adhered cells is significant, but the result is still much better than the number of adhered cells harvested by the conventional method before plating (Figures 2F and S1I). The revised passaging method is potentially beneficial for the preparation of human PSCs to be used in later applications.

One of the highest priority problems for PSC culture systems is avoiding malignant transformation by the abnormal growth of unexpected subpopulations. Thus, we created a human iPSC line overexpressing BCL2L1, which drives the strong selective advantage of chromosome 20q11.21 amplification (Amps et al., 2011; Avery et al., 2013), and tested the effects of the passaging methods on its excessive growth rate. To this end, BCL2L1-transgenic (Tg) iPSCs were added to make up 5% of the total cell number into parental iPSCs and monitored their proportions in co-culture for 6 passages. The revised passaging method significantly attenuated the growth advantage of BCL2L1-Tg iPSCs, whereas the conventional method biased amplification (Figures 3A and 3B). This observation suggests that the revised method keeps human PSCs stable during continuous culture. We validated human PSCs split 30 times over 100 days using the revised method. These cells kept expressing core PSC transcription factors, such as OCT3/4 (Okamoto et al., 1990; Scholer et al., 1990) and NANOG (Chambers et al., 2003; Mitsui et al., 2003), and displayed an affinity for r3C2LCN (Onuma et al., 2013) (Figures 3C and S3A). We confirmed that passaging with the revised method did not induce major changes in global RNA expression, the expression of PSC and differentiation-deficiency (DD) markers (Koyanagi-Aoi et al., 2013; Ohnuki et al., 2014), and global protein expression levels (Figures 3D, 3E, S3B, and S3C). The cells could differentiate into three germ layers through directed differentiation methods (Figures 3F, 3G, S3D, and S3E) and have no apparent karyotype abnormalities (Figures 3H and S3F). These data suggest that the revised passaging method is suitable for human PSC subcultures.

**DISCUSSION**

In this study, we provide a revised passaging method optimized for the xeno-free culture of human PSCs. The major change compared with the conventional method was cell dissociation before media replacement; other factors such as reagents and mechanical dissociation techniques are flexible. Since human PSCs rapidly adhere to the matrix-coated culture vessel again after replacing dissociation reagent with growth media, forced cell detachment by scraping or pipetting should be stressful (Figures 1I, S1F, and S2). However, we demonstrated that quick cell dissociation after adding growth media achieves relatively better viability (Figure 1E). Although this quick dissociation may be applicable to one-by-one treatment of cultured cells, multi-throughput experiments need a more flexible time window of the dissociation step. Using the revised method, we showed that 10 min of incubation in TrypLE at 37°C for detachment and a subsequent 60 min of incubation at room temperature did not affect the viability of human PSCs (Figure S1G). Therefore, we concluded that the revised method is suitable for the multi- or high-throughput treatment of human PSC cultures.

The continuous commingling of dissociation buffer with the growth media overnight detached human PSCs from the culture vessel, suggesting that dissociation buffer must be removed. The ideal solution for cell viability, then, is replacing the dissociation buffer-containing media with complete media after spinning the cells down. For an automated cell culture system, however, the centrifuging process is preferred to be excluded. We showed a model of the spin-free method by changing the media after plating the cells onto a culture vessel. Removing the dissociation buffer-containing media after 10 min of plating yielded 75% of cells after 18 h of plating compared to spinning the cells down before plating. While there may still be a margin for improvement, this approach is nevertheless an attractive model for PSC-based applications.

The revised passaging method significantly improved downstream applications, in particular, directed differentiation, by improving cell survival and adhesion efficiency. We note that increasing the number of cells plated can improve the results of applications even when using the conventional passaging method, in which reproducibility tends to be low. The use of ROCK inhibitor in the human PSC culture increases the cloning efficiency from <1% to ~25% (Watanabe et al., 2007). In addition, the high affinity between integrin substrates and human PSCs allows for cell viability of ~50% (Beers et al., 2012; Chen et al., 2011; Miyazaki et al., 2012). The stress-reduced passaging method shown in this study combined with the previous findings introduced above enable 90% adhesion efficiency even at clonal density. This study optimized the passaging procedure, which has been relatively ignored compared with the media and matrix in efforts to optimize xeno-free PSC cultures. That improving the passaging method was sufficient to reach a yield with >95% viability suggests that the proper combinations of available xeno-free media and recombining matrices are already effective for efficient culturing. Since our revised method simply modifies the cell dissociation condition, it can be applied to current procedures without significant modifications and will likely improve the results of basic PSC research and applications.
Limitations of the study
The protocol provided in this study is suitable for PSCs maintained on recombinant extracellular matrices that allow for rapid and efficient adhesion. This feature overcomes the issue of forcibly dislodging PSCs after adding growth media, which reduces their viability. The passaging method with the cell dissociation before adding growth media also can be used for classical culture methods that use Matrigel or feeder cells (Ellerström et al., 2007). However, since these culture systems do not have this dislodgement issue, our revised protocol has few advantages. The revised method enhances the viability of cells only during passaging and is less effective during growth (Figures S1J and S1K). Adding cell death inhibitors is still needed to improve the expansion efficiency (Chen et al., 2021; Watanabe et al., 2007).

The present study demonstrated that the revised passaging method could attenuate the growth advantage of a BCL2L1-Tg subpopulation (Figures 3A and 3B). Previous studies showed that chromosome 20 copy number increases including BCL2L1 enhance the viability of human PSCs rather than alter proliferation (Amps et al., 2011; Avery et al., 2013). However, other common karyotypic abnormalities involving the copy-number increases of chromosomes 1, 12, and 17 promote the proliferation of human PSCs (Price et al., 2021). Further studies are needed to demonstrate whether the revised passaging method is effective for such abnormal subpopulations displaying excessive proliferation.

Finally, dissociation with EDTA led to lower viability than TrypLE or AccuMax (Figures 1B, S1C, and S1D). However, as shown in Figure S1E, EDTA treatment did not produce single cells efficiently. Thus, cell viability can be underestimated because of selective single-cell counting. Therefore, we cannot conclude that there is an advantage of enzymatic treatment compared to EDTA.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2021.100155.

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AUTHOR CONTRIBUTIONS
Conceptualization, K.T., K.W., and S.Y.; methodology, K.T. and K.W.; investigation, K.T., C.O., M.N., M.I., Y.K., and Y.M.; formal analysis, K.T., C.O., M.I., and T.T.; writing, K.T., C.O., M.I., K.W., and S.Y.; supervision, K.T. and S.Y.

DECLARATION OF INTERESTS
K.T. is on the scientific advisory board of I Peace without salary, M.I. is a scientific adviser for xFOREST Therapeutics without salary, and S.Y. is a scientific advisor for iPSC Academia Japan without salary. All of the other authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-OCT3/4 | Santa Cruz Biotechnology | Cat# sc-5279, RRID:AB_628051 |
| Goat polyclonal anti-NANOG | R&D Systems | Cat# AF1997, RRID:AB_355097 |
| rBC2LCN-FITC (AiLecS1-FITC) | WAKO | 180-02991 |
| Goat polyclonal anti-SOX17 | R&D Systems | Cat# AF1924, RRID:AB_355060 |
| Goat polyclonal anti-HAND1 | R&D Systems | Cat# AF3168, RRID:AB_2115853 |
| Rabbit polyclonal anti-PAX6 | BioLegend | Cat# 901301, RRID:AB_2565003 |
| Mouse monoclonal anti-cardiac Troponin-T | BD Biosciences | Cat# 565744, RRID:AB_2739341 |
| Rabbit monoclonal anti-Phospho-Histone H2A.X (Ser139) | Cell Signaling Technology | Cat# #9718, RRID:AB_2118009 |
| Rabbit monoclonal anti-Cleaved CASPASE-3 (Asp175) | Cell Signaling Technology | Cat# #9664, RRID:AB_2070042 |
| Rabbit polyclonal anti-CASPASE-3 | Cell Signaling Technology | Cat# #9662, RRID:AB_331439 |
| Rabbit monoclonal anti-VINCULIN | Cell Signaling Technology | Cat# #13901, RRID:AB_2782768 |
| Alexa 488 Plus donkey anti-mouse IgG | Thermo Fisher Scientific | Cat# A32766, RRID:AB_2762823 |
| Alexa 555 donkey anti-rabbit IgG | Thermo Fisher Scientific | Cat# A31572, RRID:AB_162543 |
| Alexa 647 Plus donkey anti-rabbit IgG | Thermo Fisher Scientific | Cat# A32795, RRID:AB_2762835 |
| Alexa 647 Plus donkey anti-goat IgG | Thermo Fisher Scientific | Cat# A32849, RRID:AB_2762840 |
| **Chemicals, peptides, media and recombinant proteins** |        |            |
| iMatrix-511 silk | Nippi | Cat# 892021 |
| Vitronectin (20-398 aa), Human recombinant | WAKO | Cat# 220-02041 |
| StemFit AK02N | Ajinomoto | Cat# AK02 |
| TeSR-E8 | Veritas | Cat# ST-05990 |
| Y-27632 | WAKO | Cat# 036-24023 |
| Puromycin Dihydrochloride | Thermo Fisher Scientific | Cat# A1113803 |
| Dulbecco’s Phosphate Buffered Saline | Nacalai tesque | Cat# 14249-95 |
| UltraPure 0.5 M EDTA, pH8.0 | Thermo Fisher Scientific | Cat# 15575020 |
| TrypLE express | Thermo Fisher Scientific | Cat# 12604013 |
| AccuMax | Innovative Cell Technologies | Cat# AM105 |
| STEMdiff SMADi Neural Induction Kit | Veritas | Cat# ST-08581 |
| Activin A, Human recombinant | Nacalai tesque | Cat# 18585-81 |
| BMP4, Human recombinant | Peprotech | Cat# 120-05ET |
| bFGF, Human recombinant | Peprotech | Cat# 100-1BB |
| PIK-90 | Cayman Chemical | Cat# 10010749 |
| PI-103 Hydrochloride | MedChemExpress | Cat# HY-10115A |
| AB3-01 | Stergent | Cat# 04-0014 |
| LDN193189 | Stergent | Cat# 04-0074 |
| CHIR99021 | Nacalai tesque | Cat# 04-0036 |
| IWP-4 | Stergent | Cat# 04-00974 |
| DMEM/F-12 | Thermo Fisher Scientific | Cat# 10565018 |
| Glasgow’s MEM | WAKO | Cat# 078-05525 |
| RPMI1640 | Thermo Fisher Scientific | Cat# 11875-093 |
| B27 supplement | Thermo Fisher Scientific | Cat# 17504044 |
| B27 supplement, minus insulin | Thermo Fisher Scientific | Cat# A1895601 |
| Knockout Serum Replacement | Thermo Fisher Scientific | Cat# 10828028 |
| MEM Non-Essential Amino Acids Solution | Thermo Fisher Scientific | Cat# 11140050 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE                              | SOURCE                  | IDENTIFIER  |
|-------------------------------------------------|-------------------------|-------------|
| Sodium pyruvate solution                         | Sigma-Aldrich           | Cat# S8636  |
| 2-mercaptoethanol                                | Thermo Fisher Scientific | Cat# 21985023 |

### Critical commercial assays

| Human Stem Cell Nucleofector Kit 1               | Lonza                   | Cat# VAPH-5012 |
| Hoechst 33342                                    | DOJINDO                 | Cat# H342     |
| Calcein-AM                                       | DOJINDO                 | Cat# C396     |
| Propidium Iodide                                 | DOJINDO                 | Cat# P378     |
| BCIP-NBT Solution                                | Nacalai tesque          | Cat# 19880-84 |
| 0.4% Trypan blue solution                        | WAKO                    | Cat# 207-17081|
| Fixation buffer                                  | BioLegend               | Cat# 420801   |
| 4% Paraformaldehyde Solution                     | Nacalai tesque          | Cat# 09154-85 |
| 10% TritonX-100 solution                         | Teknova                 | Cat# T1105    |
| Normal donkey serum                              | Sigma-Aldrich           | Cat# D9663    |
| QiAozl lysis reagent                             | QIAGEN                  | Cat# 79306    |
| miRNAesy Mini Kit                                | QIAGEN                  | Cat# 217004   |
| RNase-Free DNase set                             | QIAGEN                  | Cat# 79254    |
| DNeasy Blood & Tissue Kit                        | QIAGEN                  | Cat# 69504    |
| ReverTra Ace qPCR RT Kit                         | TOYOBO                  | Cat# FSG-101  |
| THUNDERBIRD Next SYBR qPCR Mix                   | TOYOBO                  | Cat# OX-201   |
| TaqMan Universal Master Mix II, no UNG           | Thermo Fisher Scientific | Cat# 4440040  |
| TaqMan Assay, human RNase P                       | Thermo Fisher Scientific | Cat# 4403326  |
| KOD One Master Mix                               | TOYOBO                  | Cat# KMM-101  |
| NEBuilder HiFi DNA Assembly Master Mix           | New England Biolabs     | Cat# E2621    |
| RIPA buffer                                      | Sigma-Aldrich           | Cat# R0278    |
| cOmplete Mini EDTA-free Protease Inhibitor Cocktail | Roche               | Cat# 469319001 |
| Pierce BCA Protein Assay Kit                     | Thermo Fisher Scientific | Cat# 23227    |
| 12-230 kDa Jess or Wes Separation Module, 8 x 25 capillary cartridges | proteinsimple | Cat# SM-W004 |
| Anti-Rabbit Detection Module for Jess, Wes, Peggy Sue or Sally Sue | proteinsimple | Cat# DM-001 |
| Agilent RNA6000 Pico Kit                         | Agilent                 | Cat# 5067-1513|
| Agilent High-Sensitivity DNA Kit                  | Agilent                 | Cat# 5067-4626|
| IDT for Illumina RNA UD Indexes Set A, Ligation  | Illumina                | Cat# 20040553 |
| Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus | Illumina | Cat# 20040529 |
| NextSeq 500/550 High Output v2 Kit               | Illumina                | Cat# FC-404-2005 |
| SDB-XC Empore disc cartridge                     | 3M                      | Cat# 2340     |
| Sodium dodecyl sulfate (SDS)                     | Nacalai Tesque          | Cat# 3106-75  |
| Sodium deoxycholate (SDC)                        | WAKO                    | Cat# 190-08313|
| Sodium lauroyl sarcosinate (SLS)                 | WAKO                    | Cat# 192-10382|
| Trypsin / Lys-C Mix, Mass Spec Grade             | Promega                 | Cat# V5072    |
| Ethyl acetate                                    | WAKO                    | Cat# 051-00356|
| Acetonitrile                                     | WAKO                    | Cat# 018-19853|
| Acetic acid                                      | WAKO                    | Cat# 018-20061|
| Formic acid                                      | WAKO                    | Cat# 066-00461|
| Trifluoroacetic acid (TFA)                       | WAKO                    | Cat# 204-02743|
| Dithiothreitol (DTT)                             | WAKO                    | Cat# 045-08974|
| Iodoacetamide (IAA)                              | WAKO                    | Cat# 095-02151|

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kazutoshi Takahashi (kazu@cira.kyoto-u.ac.jp)

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Ammonium bicarbonate | WAKO   | Cat# 018-21742 |
| 1M Tris-HCl (pH 9.0) | Nippon gene | Cat# 314-90381 |
| Protease Inhibitor  | Sigma-Aldrich | Cat# P8340 |
| Phosphatase Inhibitor cocktail 2 | Sigma-Aldrich | Cat# P5726 |
| Phosphatase Inhibitor cocktail 3 | Sigma-Aldrich | Cat# P0044 |
| Aurora column (25 cm length, 75 μm i.d.) | IonOpticks | Cat# AUR25075C18AC |
| RNA-seq files       | NCBI GEO | GSE184071 |
| Mass spectrometry data files | jPOST | JPIST001327 (PXD028731) |
| Raw images and numerical data | Mendeley | DOI: 10.17832/4c7bvcvj5p.1 |
| WTB6 human iPSC line | Miyakoa et al. (2014) | RRID:CVCL_VM30 |
| WTC11 human iPSC line | Kreitzer et al. (2013) | RRID:CVCL_Y803 |
| 585A1 human iPSC line | Okita et al. (2011) | RRID:CVCL_DQ06 |
| 201B7 human iPSC line | Takahashi et al. (2007) | RRID:CVCL_A324 |
| H9 human ESC line   | Thomson et al. (1998) | RRID:CVCL_9773 |
| pAAVS1-P-CAG-GFP    | Oceguera-Yanez et al. (2016) | Addgene #80491, RRID:Addgene_80491 |
| pXAT2               | Oceguera-Yanez et al. (2016) | Addgene #80494, RRID:Addgene_80494 |
| PB-CAG-Clover-P2A-BCL2L1-IP | This study | N/A |

### Deposited data

| RNA-seq files | NCBI GEO | GSE184071 |
| Mass spectrometry data files | jPOST | JPIST001327 (PXD028731) |
| Raw images and numerical data | Mendeley | DOI: 10.17832/4c7bvcvj5p.1 |

### Experimental models: Cell lines

| WTB6 human iPSC line | Miyakoa et al. (2014) | RRID:CVCL_VM30 |
| WTC11 human iPSC line | Kreitzer et al. (2013) | RRID:CVCL_Y803 |
| 585A1 human iPSC line | Okita et al. (2011) | RRID:CVCL_DQ06 |
| 201B7 human iPSC line | Takahashi et al. (2007) | RRID:CVCL_A324 |
| H9 human ESC line | Thomson et al. (1998) | RRID:CVCL_9773 |

### Oligonucleotides

| see Table S2 | eurofins, IDT | N/A |

### Recombinant DNA

| pAAVS1-P-CAG-GFP | Oceguera-Yanez et al. (2016) | Addgene #80491, RRID:Addgene_80491 |
| pXAT2 | Oceguera-Yanez et al. (2016) | Addgene #80494, RRID:Addgene_80494 |

### PB-CAG-Clover-P2A-BCL2L1-IP

| This study | N/A |

### Software and algorithms

| Hybrid cell count application BZ-H3C | https://www.keyence.com/global.jsp | KEYENCE |
| Compass for SW6.0 | https://www.proteinsimple.com/compass/downloads/ | proteinsimple |
| STAR Aligner (version 2.5.3a) | https://github.com/alexdobin/STAR | Dobin et al. (2013) |
| bowtie 2 (version 2.2.5) | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml | Langmead and Salzberg (2012) |
| cutadapt-1.12 | http://gensof.pasteur.fr/docs/cutadapt/1.18/index.html | Martin (2011) |
| SAM tools (version 1.10) | https://sourceforge.net/projects/samtools/files/samtools/1.7/ | Li et al. (2009) |
| RSQeQC (version 4.0.0) | http://rsqec.sourceforge.net/ | Wang et al. (2012) |
| HTSeq (version 0.13.5) | https://htseq.readthedocs.io/en/master/ | Anders et al. (2015) |
| DESeq2 (version 1.26.0) | https://bioconductor.org/packages/release/bioc/html/DESeq2.html | Love et al. (2014) |
| DIA-NN (version 1.8) | https://github.com/vdemichev/DiaNN | Demichev et al. (2020) |
| Office 365 | https://www.office.com/ | Microsoft |
| Adobe Creative Cloud | https://www.adobe.com/ | Adobe |
| GraphPad Prism 8.0.2 | https://www.graphpad.com/scientific-software/prism/ | GraphPad |

### Open Access

A Self-archived copy in Kyoto University Research Information Repository: https://repository.kulib.kyoto-u.ac.jp
**Materials availability**
Unique reagents generated in this study are available from the lead contact with a Materials Transfer Agreement.

**Data and code availability**
- RNA-sequencing and proteome data are accessible in the Gene Expression Omnibus database of the National Center for Biotechnology Information website and in the Japan Proteome Standard Repository/Database, respectively. Accession numbers are listed in the key resources table. The images, numerical data and statistical analysis results that were not shown in the paper have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

The human pluripotent stem cell (PSC) lines WTB6 iPSC (P49) (RRID:CVCL_VM30), WTC11 iPSC (P46) (RRID:CVCL_Y803), 585A1 iPSC (P49) (RRID:CVCL_DQ06), 201B7 iPSC (P28) (RRID:CVCL_A324), and H9 ESC (P51) (RRID:CVCL_9773) were cultured in humidified incubators at 37°C in 5% CO₂ and 20% O₂. All reagents were warmed in a water bath set at 23°C before use unless otherwise noted. We confirmed all cell lines used in the study were negative for mycoplasma contamination by performing a periodic test (Young et al., 2010). The karyotype analysis was performed by Nihon Gene Research Laboratories, Inc. The experiments using H9 ESCs were conducted in conformity with “The Guidelines on the Distribution and Utilization of Human Embryonic Stem Cells” of the Ministry of Education, Culture, Sports, Science and Technology, Japan. WTB6 and WTC11 were provided by Bruce R. Conklin (The Gladstone Institutes and University of California San Francisco).

**METHOD DETAILS**

**Cell culture**

Human PSCs listed in the key resources table were maintained on a laminin 511 E8 fragment (iMatrix, Nippi) in StemFit AK02N media (Ajinomoto) as described previously (Miyazaki et al., 2012; Nakagawa et al., 2014). We also cultured human PSCs on recombinant human vitronectin (20-388 a.a.) (rhVTN, WAKO) in TeSR-E8 media (Veritas) (Beers et al., 2012; Chen et al., 2011). The media was changed daily.

**The revised passaging method**

In the study, after three days of seeding human PSCs at 2 x 10⁵ cells per well of a 6-well plate (~20,000 cells/cm²), we performed the passaging with the indicated method and reagent unless otherwise stated. The cells were washed once with Dulbecco’s Phosphate-Buffered Saline, calcium-free and magnesium-free (hereafter D-PBS, Nacalai tesque) and treated with a dissociating solution, such as TrypLE express (Thermo Fisher Scientific), 0.5-5 mM EDTA, which was prepared by diluting UltraPure 0.5 M EDTA (Thermo Fisher Scientific) in D-PBS, or AccuMax (Innovative Cell Technologies), for 10 min at 37°C. Then the dissociation was performed in the detachment reagent by pipetting up and down using a 1-mL micropipette or using a cell scraper (IWAKI), and the cell suspension was mixed with StemFit AK02N (~10 times volume of dissociation reagent) in a 15-mL tube. Live and dead cells were counted by mixing 1:1 with 0.4% trypan blue solution (WAKO) and using a TC20 automated cell counter (Bio-Rad). After counting the cell number, the cells were spun down at 240 xg for 5 min at room temperature, and the cell pellet was resuspended in StemFit AK02N media. A Rho associate kinase inhibitor, Y-27632 (10 μM, WAKO), and iMatrix (0.25 μg per 1 cm² of the culture vessel surface area) were added to the media during the replating (Miyazaki et al., 2017). For the culture system of TeSR-E8 media and rhVTN, the culture vessels were coated with rhVTN (0.75 μg per 1 cm²) for 1 h at room temperature before use. We routinely plated 2 x 10⁵ live cells per well of a 6-well plate and passaged them every three days.

**Conventional passaging**

The conventional passaging method was described previously (Nakagawa et al., 2014). In brief, the cells were washed with D-PBS and treated with a 1:1 mix of TrypLE express and 0.5 mM EDTA (T1E1) for 5 min at 37°C. Then we aspirated off the supernatant, added an appropriate volume of StemFit AK02N media, harvested the cells using a cell scraper, and collected them in a 15-mL tube. We dislodged the cells using a cell scraper within 30 s after adding the media unless otherwise noted. After that, we counted the cells and plated 2 x 10⁵ live cells per well of a 6-well plate in StemFit AK02N media supplemented with 10 μM Y-27632 and iMatrix (0.25 μg per 1 cm² of the culture vessel surface area). To fairly compare the two methods, we incubated the cells for 10 min rather than 5 min in TrypLE instead of T1E1 in the experiments shown in the figures except Figures 1B and 1C.
**Cell Reports Methods**

**Report**

**Cell adhesion test**
Cells harvested using the indicated dissociation protocol were plated at 2 x 10^5 cells per well of a 6-well plate by the non-coating method (Miyazaki et al., 2017). Eighteen hours after plating, the cells were washed three times with D-PBS and fixed by treating them with 4% paraformaldehyde (BioLegend) for 15 min at room temperature. Then the fixation buffer was replaced with D-PBS supplemented with 1 μg/mL Hoechst 33342 (DOJINDO) and incubated for 60 min at room temperature with protection from light. Ten images of each sample were randomly taken using a BZ-X710 all-in-one fluorescence microscope (KEYENCE), and the number of Hoechst-stained nuclei was counted using a hybrid cell count application BZ-H3C (KEYENCE).

**Single cell plating in clonal density**
The cells were harvested using the methods indicated above and serially diluted to prepare the cell suspension at 50 live cells/mL in StemFit AK02N plus 10 μM Y-27632 and iMatrix (0.25 μg per 1 cm² of the culture vessel surface area). Nine milliliters of the cell suspension containing 450 cells were transferred onto a 100-mm dish and incubated overnight at 37°C. The media was changed every other day with fresh StemFit AK02N. After 8 days of culture, the cells were fixed with 4% paraformaldehyde solution (Nacalai tesque) for 2 min at room temperature and incubated with BCIP-NBT solution (Nacalai tesque) for 60 min to visualize alkaline phosphatase positive colonies.

**Live and dead cell staining**
The cells were treated with 1 μg/mL of Calcein-AM (DOJINDO) and 2 μg/mL of propidium iodide (PI, DOJINDO) for 15 min at 37°C to discriminate between live and dead cells. After the incubation, the cells were imaged using the BZ-X710 with a 10x objective, and the number of PI positive cells was counted using the BZ-H3C.

**RNA isolation and quantitative reverse transcription polymerase chain reaction (qPCR)**
The cells were lysed with a QIAzol reagent (QIAGEN), and the total RNA was extracted using a miRNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol. One microgram of purified RNA was applied for the first-strand cDNA synthesis using a ReverTra Ace qPCR RT Master Mix (TOYOBO). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a THUNDERBIRD Next SYBR qPCR Mix (TOYOBO) and gene specific primers on a QuantStudio 3 real-time PCR system (Applied Biosystems). The raw Ct values were normalized to the housekeeping gene human ACTB via the delta Ct method, and then the relative expression was calculated as the fold-change from the control. Primer sequences are listed in Table S2.

**Antibody-based quantitative protein analysis**
Cells harvested using the conventional or revised method were pelletized by centrifugation and washed once with D-PBS. The pellets were lysed with ice-cold RIPA buffer (Sigma-Aldrich) supplemented with complete EDTA-free protease inhibitor cocktail (Sigma-Aldrich) and stored at −80°C until use. The cell lysates were heated at 95°C for 5 min, spun at 15,300 g for 15 min at 4°C, and the cleared supernatant was collected and used for the following analysis. The protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and EnVision 2104 plate reader (Perkin Elmer) as instructed. We utilized a size-based antibody-based quantitative protein analysis on a Wes automated capillary electrophoresis platform (proteinsimple) and 12-230 kDa Separation Module (proteinsimple) as instructed. We loaded 2.0-2.4 mg of cell lysates for each detection along with the following antibodies: rabbit monoclonal anti-phospho-H2AX (Ser139) (1:25, Cell Signaling Technology), rabbit polyclonal anti-CASPASE-3 (1:50, Cell Signaling Technology), rabbit monoclonal anti-cleaved CASPASE-3 (1:50, Cell Signaling Technology), and rabbit monoclonal anti-VINCULIN (1:250, Cell Signaling Technology). The data was visualized and analyzed using Compass for SW6.0 software (proteinsimple).

**Gene targeting**
The CRISPR/Cas9-mediated knock-in experiment was performed as described previously (Oceguera-Yanez et al., 2016; Takahashi et al., 2020) with slight modifications. In brief, we transfected 3 μg of pXAT2 and 7 μg of pAAVS1-P-CAG-GFP into 1 million WT6 iPSCs using a Human Stem Cell Nucleofector Kit I (Lonza) and the A-023 program of a Nucleofector Lib device (Lonza). pXAT2 encodes S. pyogenes Cas9 and a single guide RNA (sgRNA) for AAVS1 (5′-GGGGCCACTAGGGACAGGAT-3′). A promoter-less splice acceptor (SA), thosea asigna virus 2A (T2A) peptide and puromycin-resistance gene cassette in pAAVS1-P-CAG-GFP targeting vector is activated by the endogenous AAVS1 promoter only upon correct homologous recombination. Fifty thousand electroporated cells were plated onto a 100-mm dish in StemFit AK02N supplemented with 10 μM Y-27632 and iMatrix (0.25 μg per 1 cm² of the culture vessel surface area). Three days after the electroporation, we started the selection with 0.5 μg/mL puromycin and continued it until the non-transfected control died completely and the colonies of the targeted cells grew enough. The colonies were visualized by staining using BCIP-NBT solution, and the number of alkaline phosphatase positive colonies were counted. In this assay, we considered drug-resistant colonies are AAVS1-targeted clones.

**Plasmid construction**
A triple tandem repeat of Simian Virus 40 nuclear localization signal-tagged Clover (with no stop codon) and a protein coding sequence of human BCL2L1 gene linked in-frame by the porcine teschovirus-derived 2A peptide sequence were generated by
PCR using KOD One Master Mix (TOYOBO). The fragment was inserted into the Xhol site of PB-CAG-IP using NEBuilder HiFi DNA Assembly (New England Biolabs) and verified by sequencing. The resulting plasmid encodes Clover, BCL2L1 and puromycin-resistance gene driven by a constitutively active CAG promoter. The sequence can be provided upon request.

**Generation of BCL2L1-expressing iPSC line**
We transfected 7 µg of PB-CAG-Clover-P2A-BCL2L1-IP and 3 µg of pCW-hyPBase into one million WTB6 iPSCs as described previously (Takahashi et al., 2020; Yusa et al., 2011). Two days after the transfection, the cells were selected with 0.5 µg/mL of puromycin until the non-transfected cells died completely. The colonies expressing Clover uniformly were chosen and isolated.

**Cell competition assay**
We added WTB6 iPSCs expressing exogenous BCL2L1 to be 5% of total cell number into parental iPSCs. The mixed cells were plated at 2 x 10^5 cells per well of a 6-well plate and passaged every three days as described above. The cell population was analyzed by the microscopic observation of Clover fluorescence and PCR-based copy number assay for a puromycin-resistance gene.

**Genomic PCR for copy number quantification**
Genomic DNA was isolated using a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer’s protocol. Thirty nanograms of purified DNA was used for qPCR using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific) on a QuantStudio 3 Real Time PCR System. A PrimeTime qPCR Assay (Integrated DNA Technologies) was used to detect the puromycin-resistance gene in the PB-CAG-Clover-P2A-BCL2L1-IP vector, and a TaqMan Copy Number Reference Assay human RNase P (Thermo Fisher Scientific) was used as an internal control. The primer and probe sequences are provided in Table S2.

**Endoderm differentiation**
The directed differentiation into endoderm was performed as described previously with slight modifications (Loh et al., 2014; Martin et al., 2020). A day before the differentiation, human PSCs were plated at 5 x 10^5 cells per well of a 12-well plate on iMatrix in StemFit AK02N plus 10 µM Y-27632. The cells were treated with 100 ng/mL Activin A (Nacalai tesque), 3 µM CHIR99021 (Nacalai tesque), 20 ng/mL BFGF (Peprotech) and 50 nM Pl-103 (Cayman Chemical) in differentiation media 1 (DM1), which consisted of DMEM/F12 (Thermo Fisher Scientific), 2% B27 supplement (Thermo Fisher Scientific), 1% MEM Non-Essential Amino Acids (NEAA, Thermo Fisher Scientific) and 0.1 mM 2-mercaptoethanol (Thermo Fisher Scientific) for 24 h (day 1). Then the cells were cultured in DM1 supplemented with 100 ng/mL Activin A and 250 nM LDN193189 for 48 h (days 2 and 3). Two days later, the cells were maintained in DM1 containing 100 ng/mL Activin A (days 4 and 5). The media was changed daily.

**Mesoderm differentiation**
Mesoderm differentiation was performed as described previously with slight modifications (Loh et al., 2016; Martin et al., 2020). A day before the differentiation, human PSCs were plated at 5 x 10^5 cells per well of a 12-well plate on iMatrix in StemFit AK02N plus 10 µM Y-27632. The cells were treated with 1 µM A83-01 and 250 nM LDN193189 in Glasgow’s MEM (WAKO) containing 8% Knockout serum replacement (Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma-Aldrich), 1% NEAA and 0.1 mM 2-mercaptoethanol for 5 days. The media was changed daily.

**Ectoderm differentiation**
The directed differentiation into neuroectoderm was performed as described previously (Chambers et al., 2009; Doi et al., 2014; Takahashi et al., 2020). A day before the differentiation, human PSCs were plated at 5 x 10^5 cells per well of a 12-well plate on iMatrix in StemFit AK02N plus 10 µM Y-27632. The cells were treated with 1 µM A83-01 and 250 nM LDN193189 in StemFit AK02N supplemented with 10 µM Y-27632 and iMatrix (0.25 mg per 1 cm² of the culture vessel surface area). On the day we designated as day 0, the media was replaced with RPMI1640 (Thermo Fisher Scientific) supplemented with 2% B27 supplement (minus insulin) and 4 µM CHIR99021. On day 1, we removed CHIR99021. On day 3, we added 5 µM IWP-4 (Stemgent) until day 5. On day 7, we switched the media to RPMI1640 containing B27 supplement (plus insulin) and cultured the cells until day 12.
Neurosphere formation
Cells harvested using the conventional or revised method were transferred at 3 × 10^6 cells per well of an AggreWell 800 24-well plate (Veritas) in STEMdiff Neural Induction Medium supplemented with SMAD inhibitor (Veritas) and 10 μM Y-27632. Then the plate was briefly spun at 100 g for 3 min and incubated at 37°C for 18 h. After the incubation, we performed Calcein/PI staining as described above. To measure the size of the aggregates, we imaged Calcein positive cells under a 488 nm wavelength filter and measured the diameter of each aggregate using the BZ-H3C. We only evaluated single spheres in a microwell of AggreWell 800 plate.

Immunocytochemistry
Indirect immunocytochemistry was performed as described previously (Takahashi et al., 2021). The cells were washed once with D-PBS and fixed using 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized and blocked with D-PBS containing 0.1% TritonX-100 (Teknova), 1% bovine serum albumin (BSA, Thermo Fisher Scientific) and 2% normal donkey serum (Sigma-Aldrich) for 45 min at room temperature. We incubated the samples with the primary antibody diluted in D-PBS containing 1% BSA at 4°C for 6-18 h. After washing with D-PBS three times, the samples were incubated with the secondary antibody diluted in D-PBS containing 1% BSA and 1 μg/mL Hoechst 33342 for 45-60 min at room temperature in the dark. After a brief wash with D-PBS, the cells were observed using a BZ-X710. The antibodies used in the study are as follows: mouse monoclonal anti-Oct3/4 (1 μg/mL, SantaCruz), goat polyclonal anti-NANOG (5 μg/mL, R&D systems), goat polyclonal anti-SOX17 (0.5 μg/mL, R&D systems), goat polyclonal anti-HAND1 (5 μg/mL, R&D systems), rabbit polyclonal anti-PAX6 (2 μg/mL, BioLegend), mouse monoclonal anti-cardiac Troponin-T (5 μg/mL, Thermo Fisher Scientific), Alexa 488 Plus donkey anti-mouse IgG (4 μg/mL, Thermo Fisher Scientific), Alexa 555 donkey anti-rabbit IgG (4 μg/mL, Thermo Fisher Scientific), Alexa 647 Plus donkey anti-rabbit IgG (4 μg/mL, Thermo Fisher Scientific) and Alexa 647 Plus donkey anti-goat IgG (4 μg/mL, Thermo Fisher Scientific).

rBC2LCN staining
The cells were washed once with D-PBS and fixed using 4% paraformaldehyde for 15 min at room temperature. The fixed cells were incubated in D-PBS supplemented with 1/100 volume of rBC2LCN-FITC (WAKO) and 1 μg/mL Hoechst 33342 for 30 min at 37°C with protection from light. Then the cells were washed three times with D-PBS and observed using the BZ-X710.

RNA sequencing (RNA-seq) and data analysis
For the RNA-seq analysis, we used WT6 at early passage (P49, 50 and 51) and late passage (P49+30, 49+31 and 49+32) and WTC11 at early passage (P45, 46 and 47) and late passage (P45+30, 45+31 and 45+32). Cells were lysed using QIAzol reagent, and total RNA was purified as described above. Purified RNA samples were evaluated using an Agilent RNA6000 Pico Kit (Agilent) on a Bioanalyzer 2100 (Agilent). We performed the library preparation and subsequent analysis as described previously (Okubo et al., 2021a, 2021b). In brief, 100 ng of purified RNA was applied to the library construction using the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (Illumina). The libraries evaluated using an Agilent High-Sensitivity DNA Kit (Agilent) were sequenced using a NextSeq 500/500 High Output v2 Kit (Illumina). We trimmed the adapter sequence using cutadapt-1.12 (Martin, 2011), excluded reads mapped to ribosomal RNA using SAM tools (version 1.10) and bowtie 2 (version 2.2.5) (Langmead and Salzberg, 2012; Li et al., 2009), aligned the reads to the hg38 human genome using STAR Aligner (Version 2.5.3a) (Dobin et al., 2013; Wang et al., 2012), used RSeQC (version 4.0.0) for the quality check, counted the reads using HTSeq (version 0.13.5) and GENCODE annotation file (version 35) (Anders et al., 2015; Frankish et al., 2019), and normalized the counts using DESeq2 (version 1.26.0) in R (version 3.6.1) (Love et al., 2014). A Wald test was performed using the DESeq2 package.

Global proteome analysis
Cells were lysed with ice-cold PFS lysis buffer consists of 100 mM Tris-HCl, pH9.0 (Nippon Gene), 12 mM sodium deoxycholate (WAKO) and 12 mM sodium lauryl sarcosinate (WAKO) supplemented with 1% phosphatase inhibitors (Sigma-Aldrich) and 1% protease inhibitor (Sigma-Aldrich). The lysates were subjected to reduction, alkylation, Lys-C/trypsin digestion (enzyme ratio: 1/100) and desalting, as previously described (Iwasaki et al., 2019). Two-hundred and fifty nanograms of the peptides were loaded and separated on an Aurora column (25 cm length, 75 μm i.d., IonOpticks) using a nanoElute (Bruker) for subsequent analysis by timsTOF Pro system (Bruker). The mobile phases were composed of 0.1% formic acid (solution A) and 0.1% formic acid in acetonitrile (solution B). A flow rate of 400 nL/min of 2-17% solution B for 60 min, 17-25% solution B for 30 min, 25-37% solution B for 10 min, 37-80% solution B for 10 min, and 80% solution B for 10 min was used (120 min in total). The applied spray voltage was 1400 V, and the interface heater temperature was 180°C. To obtain MS and MS/MS spectra, the Parallel Accumulation Serial Fragmentation (PASEF) acquisition method with data-independent acquisition (DIA) mode was used (diaPASEF) (Meier et al., 2020). For diaPASEF settings, 1.7 s per one cycle with precursor ion scan and 16 times diaPASEF scans were conducted with the MS/MS isolation width of 25 m/z, precursor ion ranges of 400–1200 m/z, ion mobility ranges of 0.57–1.47 V s cm⁻². The obtained DIA data were searched by DIA-NN (v1.8) (Demichev et al., 2020) against selected human entries of UniProt/Swiss-Prot release 2020_03 with the carbamidomethylation of cysteine as the fixed modification and protein N-terminal acetylation and methionine oxidation as the variable modification. For the other DIA-NN parameters, Trypsin/P protease, one missed cleavage, peptide length range of 7-30, precursor m/z range of 300-1800, precursor
charge range of 1-4, fragment ion m/z range of 200-1800, and 1% precursor FDR were used. The values “PG.Normalised” from the results were used as representative protein area values for the comparison.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We show the results as individual data (colored dots) and means (red bars) represented by using GraphPad Prism version 8.0.2 (GraphPad). We performed statistical analyses including unpaired, two-tailed t-tests to calculate p-values for the difference between the sample and control using Excel (Microsoft) and one-way analysis of variance (ANOVA) for multiple comparisons (Tukey’s multiple comparisons test) using GraphPad Prism 8.0.2. The p-values less than 0.05 were considered significant and are indicated by the asterisks in the figures.