Modulating cell state to enhance suspension expansion of human pluripotent stem cells

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The development of cell-based therapies to replace missing or damaged tissues within the body or generate cells with a unique biological activity requires a reliable and accessible source of cells. Human pluripotent stem cells (hPSC) have emerged as a strong candidate cell source capable of extended propagation in vitro and differentiation to clinically relevant cell types. However, the application of hPSC in cell-based therapies requires overcoming yield limitations in large-scale hPSC manufacturing. We explored methods to convert hPSC to alternative states of pluripotency with advantageous bioprocessing properties, identifying a suspension-based small-molecule and cytokine combination that supports increased single-cell survival efficiency, faster growth rates, higher densities, and greater expansion than control hPSC cultures. ERK inhibition was found to be essential for conversion to this altered state, but once converted, ERK inhibition led to a loss of pluripotent phenotype in suspension. The resulting suspension medium formulation enabled hPSC suspension yields ±0.2-fold greater than conventional hPSC in 6 d, for at least five passages. Treated cells remained pluripotent, karyotypically normal, and capable of differentiating into all germ layers. Treated cells could also be integrated into directed differentiated strategies as demonstrated by the generation of pancreatic progenitors (NKX6.1+ PDX1+ cells). Enhanced suspension-yield hPSC displayed higher oxidative metabolism and altered expression of adhesion-related genes. The enhanced bioprocessing properties of this alternative pluripotent state provide a strategy to overcome cell manufacturing limitations of hPSC.

Significance

Efficient manufacturing is critical for the translation of cell-based therapies to clinical applications. To date, high-yield expansion of human pluripotent stem cells (hPSC) in suspension bioreactors has not been reported. Here, we present a strategy to shift suspension-grown hPSC to a high-yield state without compromising their ability to differentiate to all three germ layers. In this new state, hPSC expand to densities 5.7 ± 0.2-fold higher than conventional hPSC each passage in suspension bioreactors. High-density suspension cultures enable process intensification, cost reduction, and more efficient manufacturing. This work advances cell-state engineering as a valuable tool to overcome current challenges in therapeutic cell production and processing.

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we were guided by recent reports on the generation of naive state hPSC (21–27) which may have properties that are advantageous for manufacturing in suspension, including higher adherent single-cell survival efficiencies and accelerated adherent growth rates.

Here, we describe conditions which enable the conversion of conventional, primed hPSC to an alternative functional state characterized by significantly enhanced suspension expansion capabilities. We further demonstrate that using a well-characterized protocol, these 5i-hPSC can be directed to differentiate to pancreatic progenitors. These findings lay the groundwork for adopting cell-state conversion as a strategy to overcome current challenges in manufacturing hPSC and their derivatives for targeted cell therapy applications.

Results

hPSC Treated with 5i Have Altered Colony Morphology, Transcription Factor Expression, and Surface Marker Expression. Attempts to alter primed hPSC to resemble naive mPSC, known to exist in a stable state free from exogenous signaling (28), have incorporated various combinations of small-molecule inhibitors and genetic manipulation. We tested a protocol that has been reported to maintain naive hPSC in suspension, and to partially differentiate cells to form single-cell aggregates (21, 29). In line with published reports on naive hPSC conversion (22, 23, 27), transcription factors DNM1L, DNM3, and STELLA were up-regulated, while c-MYC and DNM3 were down-regulated in 5i-hPSC.

hPSC Treated with 5i Have Enhanced Bioprocessing Properties That Facilitate Increased Yields in Suspension Culture. We next characterized 5i-hPSC growth kinetics, ability to form aggregates in static suspension, and agitation suspension survival and expansion. In preparation for suspension expansion, the growth rates of feeder-based adherent 5i-hPSC was calculated. Higher proliferation rates were exhibited in 5i-hPSC relative to primed hPSC (Fig. 2A), leading to twice as many cells in 5i-hPSC cultures at day 6 post-seeding. Colonies formed from 5i-hPSC treatment were observed to be rounded at confluence, and a plateau stationary phase of growth was not clearly observed. Both 5i-H9 and 5i-HES2 had significantly lower exponential phase doubling times (21.1 ± 3.6 and 23.3 ± 0.7 h) than untreated hPSC (30.3 ± 4.7 and 31.2 ± 1.3 h) (SI Appendix, Fig. S24).

Next, in static suspension conditions, we compared aggregate formation characteristics of 5i-hPSC to primed hPSC. Seeded as single cells at low density (100 cells per well in 96-well plate), suspension aggregate formation efficiencies were significantly higher in 5i-hPSC than in primed hPSC (4 ± 1 and 6 ± 1 fold higher using the HES2 and H9 cell lines, respectively) (Fig. 2B). This transition to suspension culture results in a feeder-free culture system. Eliminating the feeder layer, in addition to transitioning to the 5i medium composition which contains almost completely defined components (contains purified albumin), should reduce variability between manufacturing runs.

To evaluate 5i-hPSC survival and expansion as aggregates under dynamic suspension conditions, primed and 5i-hPSC were plated as single cells in low-attachment plates that were then placed on orbital shakers or into a small-scale bioreactor. After
8 h in suspension culture, 5i-H9 formed aggregates of varying sizes under both dynamic (orbital shaker) and static control conditions. Primed H9 formed small aggregates in the static control. However, in dynamic conditions, larger, irregular, and dark clumps formed (SI Appendix, Fig. S2B), suggesting the presence of dead cells and debris. After 72 h in suspension bioreactor culture, 5i-hPSC aggregate size appeared more homogeneous and smaller than primed hPSC aggregates (average diameters of 51 ± 18 and 113 ± 43 μm, respectively) (Fig. 2C and SI Appendix, Fig. S2C). These findings suggest a greater capacity for suspension aggregate growth of 5i-hPSC before aggregate size becomes growth limiting.

The suspension growth curves for primed hPSC and 5i-hPSC had varied lag-phase timing, growth rate, and maximum cell density (Fig. 2D). Cell densities peaked at days 5–6 for primed HES2, which was used as the baseline for further comparisons. 5i-HES2 and H9 density peaked at days 7 and 8 (SI Appendix, Fig. S2D). The peak 5i-HES2 density (6.32 ± 0.9 × 10^6 cells/mL) represented a 25-fold expansion and was nearly sixfold greater than that of primed HES2 at their peak density (7.73 ± 0.3 × 10^6 cells/mL). Similar growth curves were obtained for the 5i-H9 cell line, which reached a maximum cell density of 3.5 ± 0.5 × 10^6 cells/mL. Dramatically higher proliferation rates were observed in 5i-hPSC, as represented by their average doubling times in suspension: 29.0 ± 1.3 and 34.9 ± 1.9 h for 5i-HES2 and 5i-H9, respectively, and 49.2 ± 1.2 h for primed HES2.

**Culture Optimization Enables Long-Term, High-Yield Maintenance of the Pluripotent State in Suspension.** While high cell expansion in short-term 5i-hPSC bioreactor cultures can be achieved, we observed a gradual loss of the pluripotent phenotype (OCT4+/SOX2+) (Fig. 3A), in contrast to suspension expansion of primed hPSC (16). We examined various culture parameters to identify whether loss of pluripotency was due to endogenous interactions, missing feeder-layer interactions, or one of the components in 5i medium.

In short-term bioreactor studies, we observed high pluripotent phenotype at the peak cell densities reached in primed hPSC cultures, but not at the peak densities achieved in 5i-hPSC cultures (SI Appendix, Fig. S3A). To develop a bioreactor protocol to maintain pluripotency of 5i-hPSCs, we first explored a strategy in which cells were passaged at the last day on which >80% of cells expressed a pluripotent phenotype (day 6), with the hypothesis that large aggregate size inhibits pluripotency through media limitations beyond this time point. However, we were unable to maintain a pluripotent phenotype in 5i-hPSC cultured in suspension beyond two passages with this strategy (SI Appendix, Fig. S3B). We next investigated whether optimization of dissociation timing may improve pluripotency by decreasing maximum aggregate size or increasing nutrient level per cell in culture, as has been recently suggested (30). On days 4, 5, 6, and 7 of 5i-hPSC suspension culture, the aggregates were either passaged to reduce aggregate size or diluted 1:10 in fresh medium to reduce the bulk cell density (see schematic in SI Appendix, Fig. S3C). However, after 4–7 d in both the dissociation experiment and the dilution experiment, no condition was able to maintain the pluripotent phenotype (SI Appendix, Fig. S3D). Since 5i medium maintains 5i-hPSC pluripotency in adherent feeder-based conditions, we speculated that the loss of the pluripotent phenotype was due to either the transition from adherent to suspension culture or to the loss of a specific interaction with the feeder layer.

To test our hypothesis, we designed a 96-well-plate platform to screen the effects of medium components on 5i-hPSC pluripotency in suspension and in feeder-free adherent cultures (SI Appendix, Fig. S3C). To separate the effects on pluripotency of adhesion from feeder-layer supportive factors, cells were cultured on a Geltrex-coated surface. On Geltrex, 5i-hPSC were still unable to propagate while maintaining a pluripotent phenotype (Fig. 3B, adherent “IPD” condition), suggesting that adhesion alone does not maintain pluripotency in the 5i medium. Next, we confirmed that suspension culture (low-adhesion coating) in the 96-well plate accurately replicated the kinetics of pluripotency loss (SI Appendix, Fig. S3D) in bioreactor culture, thereby providing a reliable platform to screen for critical suspension culture medium components. We performed a screen evaluating each of the components in 5i medium by either removing, doubling, or halving the concentration of each component. We observed improved feeder-free adherent maintenance of pluripotency up to removal of the ERK1/2 inhibitor (PD) from the 5i medium formulation (SI Appendix, Fig. S3E). We validated this finding in a dose–response experiment to determine the effect of PD concentration on pluripotent phenotype in both feeder-free adherent and suspension conditions (Fig. 3B). PD level was negatively correlated to pluripotent phenotype by linear regression (SI Appendix, Table S1). We next tested a number of molecules recently reported to further stabilize the naive pluripotent state to determine whether they enabled hPSC maintenance under feeder-free conditions in the presence of PD. Neither the SRC inhibitor CGP (23) nor YAP/TAZ activator LPA (25) supported the maintenance of the pluripotent phenotype in the presence of PD in suspension (SI Appendix, Fig. S4A). Interestingly, PD inhibits pluripotency in suspension, yet primed hPSC converted to a dark, heterogeneous aggregate morphology typical of primed hPSC (SI Appendix, Fig. S4B). These findings demonstrate the critical role that the ERK pathway plays in this cell-state conversion and in maintenance of pluripotency in suspension.

Next, hPSC cultured in adherent 5i conditions were treated without PD in a 4i formulation in orbital shaker suspension. “4i-hPSC” were cultured in suspension for five passages, achieving an average cell density of 5.2 ± 1.1 × 10^6 cells/mL at passage 5 while maintaining a 67 ± 9% pluripotent phenotype (Fig. 3C).
These results represent a 25 ± 5.5-fold cell expansion in 6 d that is 5.7 ± 0.2-fold greater than that achieved using primed HES2 in the same time period. hPSC cultured in 5i adherent culture for five passages and 4i suspension culture for three passages do not display karyotypic abnormalities (SI Appendix, Fig. S5A) and are able to differentiate into cells of all three germ layers (SI Appendix, Fig. S5B). To test the robustness of our 4i-hPSC system, we carried out an expansion using the H9 cell line, which in our hands is unable to expand in NutriStem medium and expands only moderately in conventional serum-replacement HES medium (~1.5-fold expansion after one passage) (SI Appendix, Fig. S6A). In suspension, 4i-H9 expanded nearly ninefold in 6 d while maintaining nearly 80% pluripotent marker expression (SI Appendix, Fig. S6B). These results show promise for the use of the 4i-hPSC culture system to enable suspension bioprocessing of hPSC lines that are not readily maintained in suspension. Suspension expansion of two additional lines, WIBR3 hESC and C1.15 hiPSC (SI Appendix, Fig. S6C), in 4i conditions led to significantly enhanced fold expansion (19.0 ± 2.0- and 21.5 ± 2.5-fold expansion, respectively) relative to primed hPSC, demonstrating that this technique is applicable to other hPSC lines including induced (i) PSC. Identifying the sources of variability in suspension performance between cell lines will guide the application of cell-state conversion strategies to improve suspension manufacturing.

**Distinct Adhesion-Related Gene Expression and Medium Utilization in Suspension in 4i-hPSC.** We hypothesized that the enhanced suspension properties of 4i-hPSC, including single-cell survival and aggregate formation, may be due to reduced expression of adhesion-related genes, which lowers the cells’ dependence on adhesion for survival and maintenance of pluripotency. To identify whether adhesion-related genes are differentially expressed between 4i-hPSC and primed hPSC, we analyzed a panel of adhesion-related genes by qPCR. Notably, several genes related to the extracellular matrix as well as a variety of integrin molecules were observed to have differential expression between 4i-hPSC and primed conditions (P > 0.05, Tukey’s test) (SI Appendix, Fig. S7). As qPCR results alone cannot predict gene expression, we validated select pluripotency-associated differentially expressed genes by flow cytometry. We identified elevated expression of adhesion-related gene ICAM1 in 4i-hPSC relative to primed hPSC (Fig. 4A). Primed E-CAD expression was observed to be bimodal, with a tight E-CADhigh population. hPSC treated with 4i expressed a wider, unimodal E-CAD distribution between the primed E-CADDlow and E-CADhigh populations (Fig. 4B). Additional characterization of the interaction between cell state and culture conditions for these and other correlated markers could supplement characterization of causal factors in supporting this altered pluripotent state (29).

Next, we compared the metabolic demand and activity of 4i-hPSC to primed hPSC by comparing oxygen consumption rate (OCR) (Fig. 4C) and metabolite levels in the medium over 6 d of suspension culture. Glucose levels dropped from their initial level in all conditions, reaching a steady level by day 2 in primed conditions but not in 4i-hPSC. While 4i-hPSC continued to grow to higher densities even at lower glucose levels, the steady glucose levels in primed hPSC suggested that primed cells may require higher glucose levels for growth. Indeed, glucose uptake per cell was much lower for 4i-hPSC than primed cells, although differences in specific uptake between primed media suggest that nutrient availability may contribute to these trends. In contrast to primed conditions, 4i-culture, glutamine and glutamate dropped after day 3 at the same rate as cell expansion (SI Appendix, Fig. S8). Lactate production varied independently of cell state, while ammonium levels were highest in 4i-hPSC cultures (SI Appendix, Fig. S8). These metabolite trends lead to an elevated oxygen consumption rate of 4i-hPSC (and 5i-hPSC) relative to primed (Fig. 4C). These altered-state hPSC therefore display a metabolic usage distinct from that of primed hPSC that appears independent of base nutrient differences between the conditions.

**Directed Differentiation of 4i-hPSC to Pancreatic Progenitors Does Not Require Repriming.** We next tested the capacity for alternative-state hPSC to differentiate to pancreatic progenitors, a cell type of significant clinical interest. Directed differentiation to pancreatic progenitors from hPSC is achieved by the staged addition of molecules intended to mimic development (31) (SI Appendix, Fig. S9A). Our initial attempts to differentiate adherent 5i-hPSC (Fig. S4) and recent reports on differentiation of

![Fig. 4.](image)

**Fig. 4.** Differential ICAM1 and E-CAD expression and oxygen consumption rate (OCR). (A) ICAM1 expression is elevated in 4i relative to primed HES2. (B) Primed E-CAD expression is bimodal, with a tight distribution of the high population. 4i expression is more widely distributed. (C) Relative OCRs of 4i and 5i-HES2 and primed HES2, as measured by the MitoXpress Xtra OCR assay. OCR is normalized to primed. * indicates P < 0.05 by t test. Error bars represent SD.

![Fig. 5.](image)

**Fig. 5.** 4i-hPSC can be directed to differentiate to pancreatic progenitors without repriming. (A) Differentiation of primed hPSC results in a high (>95%) percentage of definitive endoderm marked by c-kit/cxcr4 expression after 3 d in an adherent differentiation. 12% of differentiated 5i-hPSC express this phenotype at day 3 of differentiation, whereas 5i-hPSC reprimed in serum replacement medium expressed this phenotype at 58% purity (representative experiment shown). (B) In suspension-optimized conditions, 4i-hPSC differentiate to a definitive endoderm phenotype at levels reaching 95% after 3 d. 4i-hPSC and primed hPSC can differentiate to (C) definitive endoderm and (D) pancreatic progenitors with comparable cell yields and purities. No significant difference was observed at day 3 in fold expansion and yield. Primed hPSC had significantly higher purity (P < 0.005, Tukey’s test). At day 12, no significant difference was observed in purity or yield, although 4i-hPSC had significantly higher fold expansion (P < 0.01, Tukey’s test). Error bars in this figure represent SD.
naive hPSC (32, 33) suggested that current differentiation protocols for primed hPSC result in low purity or cell yield when applied to 5i-hPSC. We hypothesized that 5i-hPSC could be differentiated to clinically relevant lineages by culturing 5i-hPSC in primed hPSC conditions before differentiation. Indeed, we found that “reprogramming” adherent 5i-hPSC for 1 d before differentiation increased the fraction of definitive endoderm cells at day 3 of differentiation (Fig. 5A).

We next sought to identify conditions that would enable suspension differentiation. While a 2-d reprogramming strategy enabled suspension differentiation of 5i-hPSC, we found that our 4i-hPSC formulation could be efficiently differentiated toward pancreatic progenitors without a reprogramming step. Both 2-d reprogramming with Nutristem feeder-free medium as well as 4i-hPSC conditions resulted in high-purity (>90%) definitive C-KIT/CXCR4 endoderm phenotype after 3 d (Fig. 5B and C) with the capacity to generate PDX1/NKX6.1 endocrine pancreatic cells after 12 d (Fig. 5D). Comparing outcomes from these strategies did not result in statistically significant differences in purity, expansion, or yield after 3 d of definitive endoderm differentiation, although 4i-hPSC had a significantly greater number of cells after 12 d of differentiation relative to reprogrammed 5i-hPSC (P < 0.01, Tukey’s test), with no significant difference in purity. 4i-hPSC are thus capable of pancreatic progenitor differentiation.

**Discussion**

Our study demonstrates that culture conditions may be manipulated to generate pluripotent states that can overcome bottlenecks in manufacturing of hPSC and their differentiated derivatives. Improved growth and maintenance of 4i-hPSC in suspension is mediated by increased shear tolerance and altered aggregation properties that promote efficient suspension colony formation leading to faster growth rates and higher achievable maximum cell densities. 4i-hPSC thus represents a more manufacturable pluripotent state characterized by the formation of a larger number of smaller aggregates which grow faster and are less susceptible to bioreactor shear-induced cell death over multiple passages in suspension, while retaining directed differentiation capability. The manufacturability of 4i-hPSC can be compared to published hPSC suspension expansion in SI Appendix, Fig. S9B.

Metabolic analysis of suspension 4i-hPSC suggests bivalent glycolytic and oxidative metabolism. 4i-hPSC had lower glucose consumption and lactate secretion per cell than primed hPSC, despite their faster growth rates, representative of the more efficient energy usage characteristic of oxidative metabolism (34). Interestingly, while glucose levels decline steadily from the outset, glutamine levels decline (and ammonium levels rise) most notably after day 4, suggesting preferential utilization of glucose in 4i-hPSC. These observations are consistent with recent reports that the pathways for bivalent metabolism are present in the naive state (22) but that glycolytic metabolism is preferred (35). Ultimately, however, metabolic pathway usage is dependent on nutrient availability (36), as is evident both by the shift to glutamine usage when glucose concentration is reduced and by the distinct metabolic profiles observed between the two primed medium compositions used (SI Appendix, Fig. S8). While this metabolic shift appears to begin at day 4 of culture, cells can continue to grow and maintain pluripotency for at least two more days of culture (Fig. 3E). This finding suggests that significant opportunities exist for improving suspension yields through medium optimization to promote pathways that drive expansion without loss of pluripotency. Strategies could include enriched medium for high-density culture and modified medium exchange methods or rates.

The hypothesis that cellular conversion to alternative pluripotent states could overcome manufacturing challenges guided the development and optimization of the 4i-hPSC suspension system. The extent to which these improvements can be attributed to modulation of cell state depends on how cell state is defined. To date, many efforts to generate a naive hPSC state are deemed successful based solely on similar gene expression profiles to naive mouse PSC. In a manufacturing context defining cell state by gene expression and purported similarity to a developmental cell state in mice is less relevant than functional definitions of alternative cell states. In this regard, the 4i-hPSC culture has clearly enabled an alternative pluripotent state that is highly amenable to high-yield suspension culture, both in bioprocess-related cellular properties and in energy-efficient metabolic pathway usage.

We hypothesize that the more robust suspension aggregation properties of 4i-hPSC (Fig. 2B and C) could have been caused by altered adhesion-related gene expression between the cell states (Fig. 4A and B). The role of ICAM1 in pluripotency is not well described. We observed elevated expression of ICAM1 in 4i-hPSC (Fig. 4A). While implicated in reprogramming to pluripotency (29), it remains to be seen if ICAM1 is causative of aggregation properties observed in 4i-hPSC or if it arises as a result of altered cell-adhesion patterns associated with this cell state. Primed E-CAD expression has been observed by Xu et al. (37) to be bimodal. They suggested that E-CAD<sup>high</sup> cells had poor suspension survival because of a lack of E-CAD–mediated cell-cell adhesion, whereas E-CAD<sup>low</sup> cells have greater potential to survive post-dissociation. They did not observe this dependence in mouse ESC or hESC with ERK1/2 and p38 inhibitors due to different regulated stability of E-CAD leading to increased adhesion. Comparing primed and 4i expression of E-CAD in our study further supports different mechanisms of suspension survival between conditions (Fig. 4B). Tightly controlled, high expression of primed E-CAD suggests this protein may be important for proliferation and survival of primed hPSC. In contrast, we hypothesize that the broad spread of E-CAD in 4i-hPSC suggests that cell survival may be less linked to E-CAD, since high expression is not necessary. Indeed, 4i treatment reduced suspension passing-related aggregation challenges and cell death (Fig. 2B and C).

Not surprisingly, analyzing published reports of suspension expansion of mouse and hPSC (SI Appendix, Fig. S9B) illustrates that 4i-hPSC are distinct from traditional hPSC cultures in suspension expansion yield, intensification (i.e., cell density), and growth rates, and are more in line with mouse PSC. Encouragingly, this relatively unoptimized bioprocess could be further enhanced by incorporating perfusion feeding schemes, medium optimization, and additional suspension media to improve the alternative cell state. Media optimization around endogenous and exogenous factors and aggregation control could prove instrumental in reducing variability in long-term suspension expansion of 4i-hPSC.

Interestingly, ERK inhibition, which blocks FGF4 differentiation signals and establishes the naive state (28), led to a loss of the pluripotent phenotype in suspension but not adherent 5i-hPSC on feeders. Removal of ERK inhibition maintained a pluripotent phenotype in suspension without diminishing the cells’ preferred suspension bioprocessing capabilities. ERK appears to cause an exit from pluripotency through a mechanism unique to the suspension environment. Adhesion and growth factor signals are thought to be integrated by RAC-facilitated formation of two distinct signaling complexes: ERK-MEK1 in adherent culture, and ERK-MEK2 in suspension culture (38). We hypothesize that the extended culture in adherent conditions in the presence of ERK, converted cells to an alternative, high-suspension-yield attractor state that is not exited upon removal of ERK, in suspension culture. The use of transient cues to acquire an attractor state has been well established in the PSC field largely through the transient overexpression of factors inducing reprogramming of somatic cells to pluripotency (39). Further study of these mechanisms may determine whether suspension culture in the presence of PD is possible or beneficial.

Combining directed differentiation protocols with the high-yield 4i-hPSC expansion system could form the basis for efficient expansion-differentiation strategies for manufacturing various
therapeutic cell types. While we observed that ERK inhibition hampered directed differentiation of 5i-hPSC, transferring to 4i or primed medium for a short period before initiating an established differentiation protocol enabled this differentiation. The outcomes using the 4i-hPSC bioprocess exceed those observed in any hPSC suspension expansion system, and are within the range of high-yield mouse PSC systems (SI Appendix, Fig. S9B). Importantly, 4i-hPSC could enable scalable high-yield production of pancreatic progenitors, which are a key intermediate cell in generating islet cells for transplantation therapies targeting type I diabetes. Our results challenge cell-state conversion as a key component to overcome manufacturing challenges in novel cell therapy bioprocessing strategies (40).

Methods and Materials
Primed hPSC were cultured on Geltrans-coated plates in Nutristem hESC Culture Medium. Where noted, hPSC were cultured on irradiated mouse embryonic fibroblast feeders in serum replacement medium (DMEM/F12, knockout serum replacement, Glutamax, nonessential amino acids, penicillin-streptomycin, and FGF2). To convert hPSC to an alternative pluripotent state, primed hPSC cultured on feeder layers were treated as described previously with modifications recommended by Hanka (27). In brief, base medium contained DMEM/F12, knockout serum replacement, Glutamax, nonessential amino acids, penicillin-streptomycin, Albumax 2, N2 supplement, and Ficoll. This medium was supplemented with UT, TGFβ1, FGF2, CHIR99021, SP600125, BIBR796, GO6983, and PD0325901 where noted. Dynamic suspension cultures were carried out in six-well plates (coated overnight with 5% Pluronic F-68) on an orbital shaker and bioreactor runs were performed using the Micro-24 bioreactor system (Pall Corporation). hPSC were seeded at a density of 2 x 10^4 cells/mL in either NS medium, SR medium, or our treatment formulation supplemented with Y-27632. Half of the medium was exchanged daily starting from day 2. Treated and untreated cells were differentiated toward endoderm, mesoderm, and ectoderm using staged differentiation protocols.

RNA was extracted for qPCR using the RNeasy Mini Kit, and reverse transcribed using the SuperScript II kit according to manufacturer’s instructions. Analysis was performed using the delta-delta Ct method, normalizing to GAPDH and primed expression level. Metabolite usage was determined from cell supernatants, and oxygen consumption rate was measured using the Mitoxpex Xtra oxygen tracker kit. G-Band karyotyping was performed by WiCell.

Parametric tests were used to analyze qPCR, doubling time, and maximum density experiments, with the Student’s t test used for two treatment experiments and Tukey’s test used for experiments with three or more treatments. A nonparametric test (Kruskal–Wallis) was used for colony formation experiments. * signifies P < 0.05 unless otherwise noted. The linear regression model was developed in Excel.

Details are found in SI Appendix, Supporting Methods.

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