Supporting Methods

Human pluripotent stem cell culture and induction to naïve-like state

We obtained H9 hESC from the WiCell Research Institute,HES2 hESC from G. Keller (McEwen Centre for Regenerative Medicine/University Health Network), and WIBR3 and C1.15 GFP lines from the Weizmann Institute (Rehovot, Israel)(29). The HES2 and H9 cells were cultured on Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Geltrex, Life Technologies) coated plates. To coat the plates, they were incubated with a 1:50 dilution of Geltrex in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) for 30 minutes at 37°C or overnight at 4°C. The H9 and HES2 hESC were seeded on the Geltrex-coated plates in Nutristem hESC XF Culture Medium (NS, Biological Industries) supplemented with 1x Penicillin-Streptomycin (Life Technologies). Where noted, hESC were cultured on feeder layers of irradiated mouse embryonic fibroblasts in serum replacement medium (SR) comprised of DMEM/F12 (Life Technologies), 20% Knockout Serum Replacement (Life Technologies), 1x Glutamax (Life Technologies), 1x Non-Essential Amino Acids (Life Technologies), 1x Penicillin-Streptomycin, and 10ng/mL FGF2 (Peprotech). We used TrypLE Express (Life Technologies) to passage the hESC as single cells at a split ratio of 1:12 to 1:24 every 5 to 6 d. All cell line stocks tested negative for mycoplasma contamination.

To convert H9 and HES2 hESC to an alternative pluripotent state, we passaged primed hESC onto feeder layers of irradiated mouse embryonic fibroblasts in medium as described previously(29) with modifications recommended by J. Hanna. In brief, base medium contained DMEM/F12, 2% Knockout Serum Replacement, 1x Glutamax, 1x Non-Essential Amino Acids, 1x Penicillin-Streptomycin, 2.5mg/mL Albumax 2 (Gibco), 1x N2 Supplement (Gibco), 0.625µL/mL Insulin (Sigma), and 50 µg/mL Ascorbic acid (Sigma). We supplemented this base medium with 20ng/mL human Leukemia Inhibitory Factor (LIF, Peprotech), 2ng/mL TGFβ1 (R&D), 8ng/mL FGF2, 0, 0.1, or 1µM PD0325901 (Reagents Direct), 3µM CHIR99021 (Reagents Direct), 5µM SP600125 (Santa Cruz Biotechnology or Stem Cell Technologies), 2µM BIRB796 (Cayman Chemical or Stem Cell Technologies), and 1µM Gö6983 (Santa Cruz Biotechnology or Stem Cell Technologies). Base media was prepared in 500mL batches and aliquoted to 15mL centrifuge tubes (Sarstedt) filled with 15mL basal media and stored at 4^0C for up to 2 weeks. Cytokines, small molecules, insulin, and ascorbic acid were added fresh to a basal media aliquot each day. Medium that is not aliquoted as described and made fresh may not perform as described in suspension culture.

Bioreactor and Suspension Culture

Dynamic suspension cultures were carried out in 6 well plates (Costar) on an orbital shaker and bioreactor runs were performed using the Micro-24 bioreactor system (Pall Corporation). To prevent cell attachment, we pre-coated suspension culture plates with 5% Pluronic F-68 (Thermo Fisher) for 30 minutes at 37°C or overnight at 4°C. Single cell dissociation was performed as described above using TrypLE Express treatment for 5 minutes at room temperature for naïve hPSC and at 37°C for control primed hPSC. We seeded hPSC at a density of $2*10^5$ cells/mL in either NS medium, SR medium, or our treatment formulation supplemented with 10µM Y-27632 (Reagents Direct) under normoxic (i.e. 21% O_2) conditions.

Two days after seeding (unless otherwise noted), we started performing daily 50% medium exchanges. Plates were placed at a 45° angle to settle aggregates at the bottom edges of each well for 3 minutes. Half of the spent medium was then removed from the culture surface and replaced with fresh medium. At the end of the culture period, aggregates were harvested and dissociated using a 5 minute TrypLE treatment at 37°C and the cells were counted on a hemocytometer using a Trypan Blue viability exclusion stain (Thermo Fisher).

Shear Sensitivity Assay

To assess shear sensitivity, cells in adherent cultures were dissociated and seeded at a density of $5*10^5$ cells/mL into Pluronic-F68 coated-plates in the presence of Y-27632 as described above. Cultures were carried out for 8 hours at an agitation rate of 90 RPM. "No-shear" controls were prepared similarly but incubated at 37°C in static conditions. After 8 hours, images of cells were taken.

RNA Preparation and Analysis

We performed RNA extraction using the RNeasy Mini Kit (Qiagen). Reverse transcription was conducted according to manufacturer's instructions (Superscript II kit, Invitrogen) using 1 µg of total RNA for each sample. Gene expression analysis was performed on an Applied Biosystems 7900 HAT Real time PCR machine using Sybr Green PCR master mix (Roche, Sigma). The cDNA of cells at inoculation (day 0) or of adherent primed hPSC was used as a relative reference using the delta-delta Ct method and expression levels of genes of interest were normalized to GAPDH expression.

Differentiation Protocols

Differentiation to ectoderm was conducted as previously described(54): Briefly, hPSC were cultured in Pluronic f-68 coated 6-well plates at a density of $0.5*10^5$ cells/mL in DMEM medium supplemented with 5% Knockout Serum Replacement, 0.1mM non-essential amino acids, 1mM sodium pyruvate (Thermo Fisher), 1% Penicillin-Streptomycin and 0.1mM β-mercaptoethanol (Sigma). At day 3, we performed a 70% medium exchange. At day 5, we transferred the aggregates to Geltrex coated tissue culture treated 6-well plates and incubated for 5 days in media comprised half DMEM-F12 and half Neurobasal medium (Thermo Fisher) supplemented with B27 without Retinoic Acid (Thermo Fisher) and N2 supplements (Thermo Fisher), 0.005% bovine serum albumin (Sigma), 1mM sodium pyruvate before staining for β-III-TUBULIN expression (Cell signaling).

Mesoderm/cardiac differentiation was conducted as previously described(55) with select modifications. Briefly, hPSC aggregates were formed by seeding a single cell suspension of hPSC at $1.2*10^6$ cells per well into a Pluronic f-68 coated 6 well-plate on an orbital shaker with 10µM Y-27632 in NS medium (Biological Industries). After 2 days, a full medium exchange was performed to a cardiac differentiation base medium of StemPro (Thermo Fisher) supplemented with 1x Glutamax, 50 µg/mL Ascorbic acid, 1% Penicillin- Streptomycin, 150 µg/mL Transferrin (Sigma-Aldrich), and 0.04 µL/mL monothioglycerol (Sigma-Aldrich). Medium exchanges were performed at days 1, 4, 8, and 12, with different cytokine cocktails at each medium exchange that corresponded with specific stages of cardiac development. Cytokines cocktails were as follows: 5ng/mL bFGF, 10ng/mL BMP4 (R&D Systems), and 3ng/mL Activin A(R&D Systems) at

Day 1; 10ng/mL VEGF (R&D Systems) and 2 μM IWP2 (Reagents Direct) at Day 4; and 10ng/mL VEGF and 5ng/mL bFGF at days 8 and 12. Cells were cultured under hypoxic conditions (5% O_2) from day 0 to 12 and under normoxic conditions from day 12-16. Aggregates were dissociated with TrypLE at day 16 and stained for cardiac TROPONIN-T (Thermo Fisher antibody MS295P).

Endoderm and pancreatic progenitor differentiation followed a variant of a published protocol(30). Briefly, pluripotent aggregates formed from hPSC seeded into suspension at $2*10^6$ cells/mL and cultured for 3 days in NS media (as described above, unless otherwise noted) were washed with phosphate buffered saline (Thermo Fisher) and cultured for 3 days in a basal medium of RPMI 1640 (Thermo Fisher), 1x Glutamax, 1%Pen/Strep, $7.8 *10^{-3}$ μL/mL monothioglycerol, and 3μL/mL "SFD". "SFD" contains 75% Iscove's Modified Dulbecco's Medium (Thermo Fisher) and 25% F12 (Thermo Fisher), supplemented with 1x N2 supplement, 1x B27 without Retinoic Acid, 25μL/mL 20% Bovine Serum Albumin solution (Wisent). At day 0 of differentiation, 2nM CHIR99021 and 100ng/mL Activin A (produced in-house) were added to this medium. On day 1 and 2, 5ng/mL bFGF, 100ng/mL Activin A, and 50μg/mL Ascorbic acid were added. Medium exchanges were performed daily. At day 3, cells were stained for C-KIT (BD Biosciences) and CXCR4 (BD Biosciences) protein expression and analyzed by flow cytometry. From day 3 to 12, the basal differentiation medium used consisted of DMEM, 1x Glutamax, 1% Pen/Strep, 1% B27 without Retinoic Acid, 1x sodium pyruvate. On day 3 and 4, the base medium was supplemented with 50ng/mL FGF10 (R&D Systems). On day 5 and 6, the base medium was supplemented with 0.25μM KAAD-cyclopamine (Toronto Research Chemicals), 50ng/mL FGF10, 2μM Retinoic Acid (Sigma-Aldrich), 100nM PDBu (Cedarlane), 200 nM LDN-193189 (Reagents Direct), and 50ug/ml Ascorbic acid. Starting on day 7 the medium was supplemented with 50ng/mL EGF, 10mM nicotinamide (Sigma-Aldrich), 50ng/mL NOGGIN, 50ug/mL ascorbic acid. Medium was changed every second day. At day 12, cells were harvested and stained for NKX6.1 (DSHB) and PDX1 (R&D Systems) protein expression.

Cell Staining, Flow Cytometry, and Immunocytochemistry

For surface staining, dissociated cells were resuspended in Hank's Buffered Saline Solution (HBSS, Thermo Fisher) supplemented with 2% FBS (Gibco) and incubated with CXCR4, C-KIT, and CD24 (BD Biosciences) antibodies at a 1:100 dilution for 30 minutes. Cells were then washed and resuspended in HBSS and 7AAD (Thermo Fisher) at a 1:1000 dilution.

For intracellular staining, dissociated cells were fixed with 4% paraformadehyde (Electron Microscopy Sciences) for 10 minutes and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). Cells were then stained with the primary antibody (OCT4, BD Biosciences; SOX2, R&D; FOXA2, Abnova; cTNT, Thermo Fisher; TUBB3, Cell Signaling; PDX1, R&D; NKX6.1, DSHB) for 25 minutes followed by the secondary antibody for 25 minutes at 4°C. Stained cells were analyzed on a FACS Canto II (BD) or FACS Fortessa (BD) flow cytometer.

For immunocytochemistry, samples were prepared as described for intracellular staining and imaged on an EVOS microscope (Thermo Fisher).

Growth Rate Calculations

Growth rates were calculated by subtracting the initial cell density from the logarithm of the final density and dividing this value by the duration of the exponential growth phase.

Medium Utilization Rate Calculations

Medium samples (1 mL) were collected and frozen immediately prior to feeding suspension cultures. Samples were thawed and analyzed using a Bioanalyzer (Nova Biomedical). Apparent metabolic rates (q_{App}) were calculated at each time point (t) collected based on the concentration (c) of each metabolite one day after a half medium exchange and the average viable cell density (VCD) at that time point:

$$q_{App} = \frac{C_t - \frac{C_0 + C_{t-1}}{2}}{(T_t - T_{t-1}) * (\frac{VCD_t + VCD_{t-1}}{2})}$$

Viable cell density was estimated based on a 1 day lag phase post-seeding followed by exponential expansion to the cell density measured at day 6 of expansion. C_0 is the concentration of metabolite in fresh medium added at each half medium exchange. To account for the presence of glutamax, which degrades into glutamine in the presence of dipeptidases secreted by cells, C_0 of glutamine and glutamate was set as the maximum concentration of these metabolites when calculating metabolic rates. The limitation of this concentration and density averaging approach is that it linearly approximates exponential growth.

Oxygen consumption rate was measured using the MitoXpress Xtra oxygen tracker kit (Luxcel Biosciences) according to manufacturer instructions.

Karyotype Analysis

hPSC were cultured in 5i medium for 5 passages and suspension 4i medium for 3 passages. Aggregates were dissociated and seeded into adherent conditions for karyotype analysis. G-Band karyotyping was performed by WiCell.

Statistical Analysis

Statistical analysis was performed using the JMP software (SAS). Parametric tests were used for qpcr, doubling time, and maximum density experiments, with the Student's T-test used for 2 treatment experiments and Tukey's HSD test used for experiments with 3 or more treatments. A non-parametric test (Kruskal-Wallis) was used for colony formation experiments since this assay was developed specifically for this study. Literature does not exist supporting an expected distribution of hPSC suspension colony formation efficiency, and a normal distribution was not assumed. * signifies $p<0.05$ unless otherwise noted. The linear regression model was developed in Excel (Microsoft).

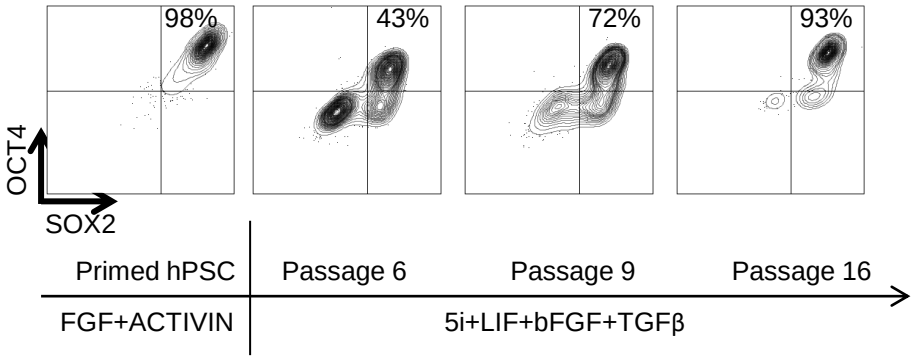
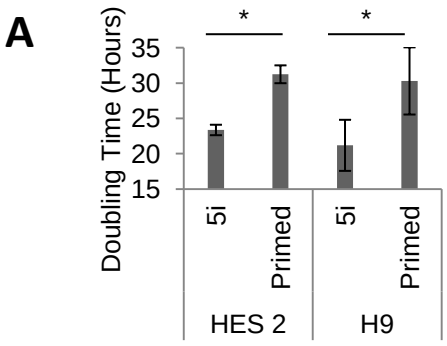
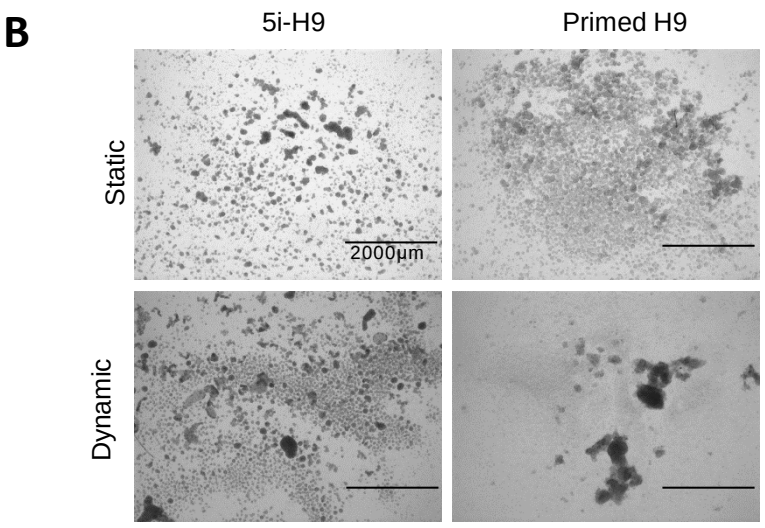
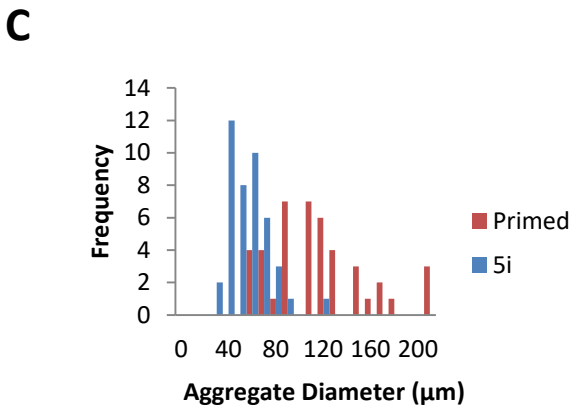


Figure S1: 5i-hPSC display a stable OCT4/SOX2 protein expression phenotype over multiple passages.







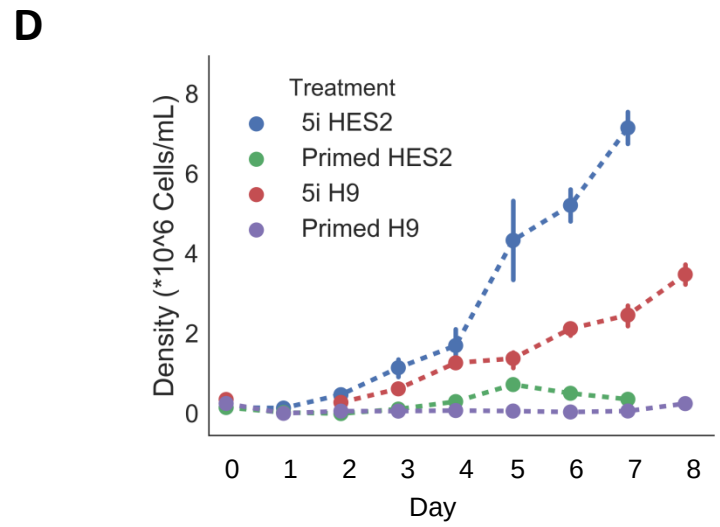
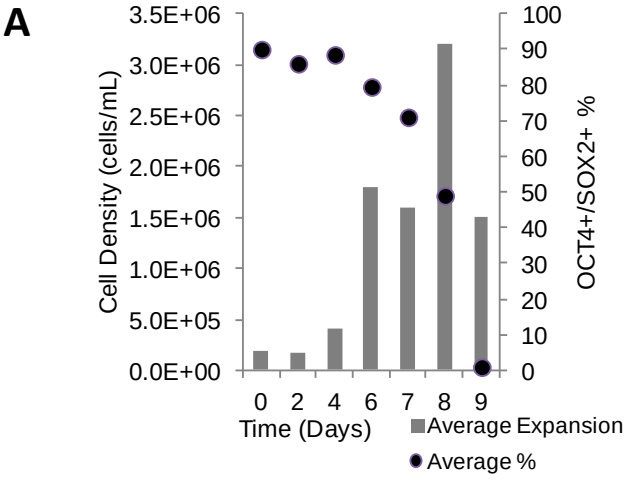
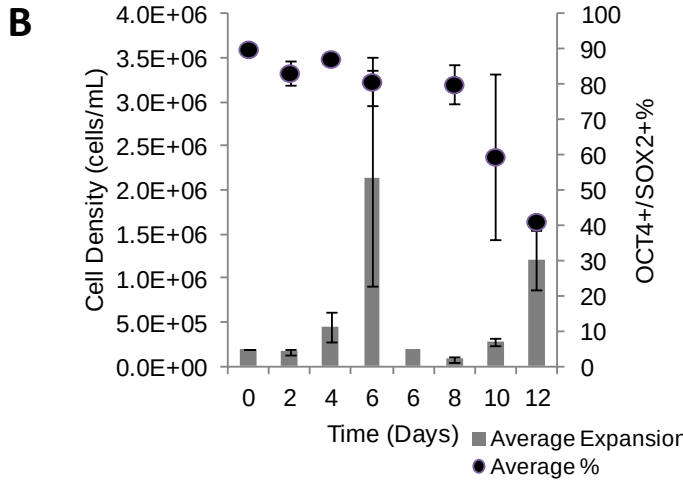
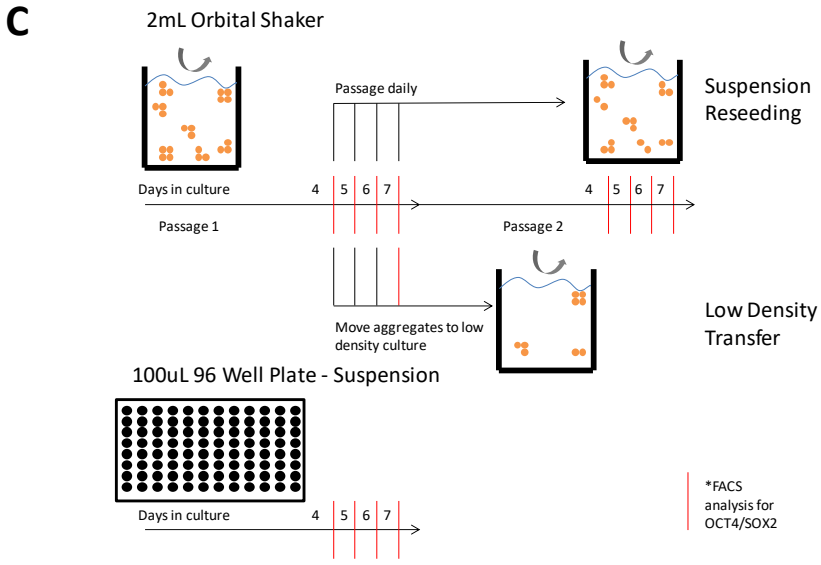
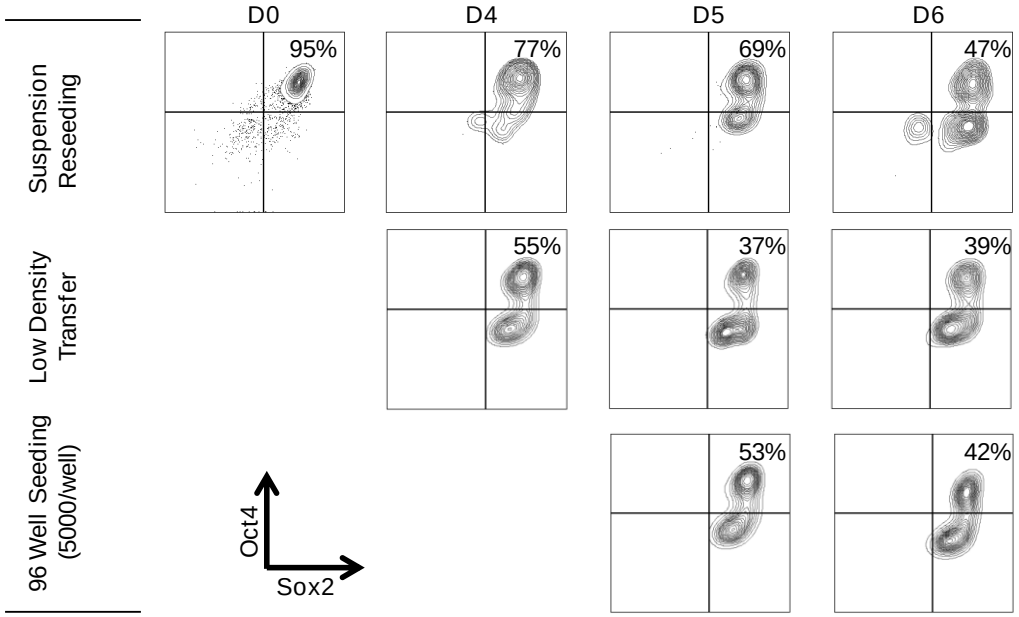


Figure S2: A) Adherent doubling times of 5i-hPSC were significantly higher than primed hPSC ($p<0.05$, Student's T-Test, n=3. Error bars represent standard deviation.) B) Primed H9 and 5i-H9 cultured in static suspension conditions for 8 hours form aggregates and do not display accumulation of debris. 5i-H9 in dynamic suspension cultures display robust aggregate formation. In primed H9 dynamic suspension cultures, debris and large, dark non-viable cell clumps were observed. C) Aggregate size distribution of Primed hPSC (red) and 5i-hPSC (blue) after 3 days in orbital shaker (dynamic) suspension conditions showing smaller aggregate sizes and size distribution in 5i treated cells. D) Suspension growth curve of primed and 5i-hPSC. Error bars represent the standard deviation.









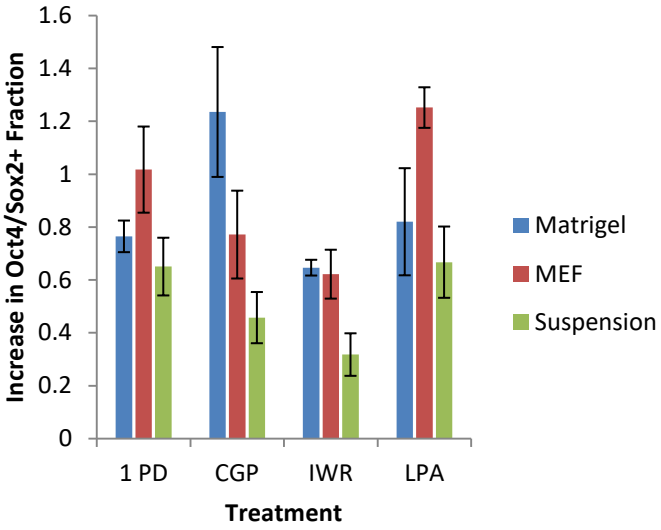
E

	2x	1x	0.5x	0x
LIF	24	38	29	34
FGF2	22	31	35	20
TGFb	12	27	26	9
CHIR	10	33	11	17
PD	2	23	3	75
SP	11	31	7	10
BIRB	2	29	5	12
GO	15	20	11	5
RI	4	16	7	14

Primed	74

Figure S3: A) Pluripotent marker expression levels are high in suspension 5i-hPSC for 3 days before 5i-hPSC levels decline over 9 days in suspension. Cell density peaks at day 8; however, pluripotent marker expression has already begun to decline. (Representative data shown) B) Passaging 5i-hPSC at a day 6, a time point at which a high level of pluripotent marker expression is observed and the culture is still in exponential growth, did not enable maintenance of the pluripotent phenotype in the second passage. Error bars represent standard deviation. C) Schematic of experiment to determine if time of passaging or density at end of culture were responsible for loss of phenotype in 5i-hPSC. In this experiment, at days 4, 5, 6, and 7 of suspension expansion, aggregates are either dissociated (suspension reseeding) or separated for low density culture without dissociation (low density transfer). Schematic of 96 well plate setup for screening critical process parameters. D) OCT4/SOX2 plots showing that dissociation followed by suspension reseeding as well as low density aggregate transfer did not rescue the pluripotent phenotype, regardless of timing. 96 well plate format recapitulates the results seen in 6 well plates. E) 5i medium component screening experiment to evaluate effects on the expression of the pluripotent phenotype (%OCT4/SOX2 +). Each value in the table refers to the concentration of the corresponding component. One factor at a time screening strategy involved doubling, halving, or removing each component.

A



B

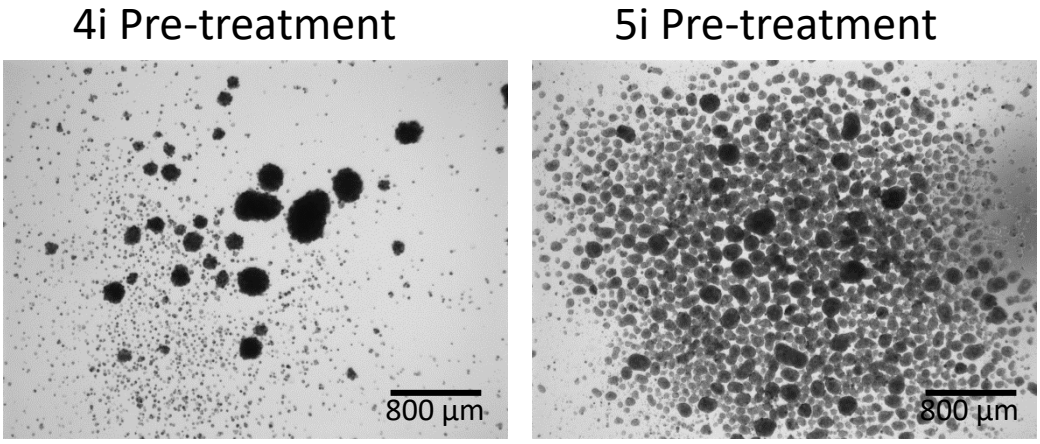


Figure S4: Additional Pathway inhibition does not enable suspension culture in the presence of ERK inhibition. A) Neither YAP/TAZ activation (LPA), SRC inhibition (CGP) nor Axin stabilization (IWR) increases the level of cells expressing the pluripotent phenotype in suspension. The OCT4/SOX2+ fraction is normalized to the 0PD (4i) condition. Error bar represent standard deviation. B) Primed hPSC treated with 5i in adherent conditions form a large number of small aggregates when cultured in suspension in 4i. Primed hPSC treated with 4i (no PD) in adherent conditions form a small number of large, dark aggregates in suspension in 4i.

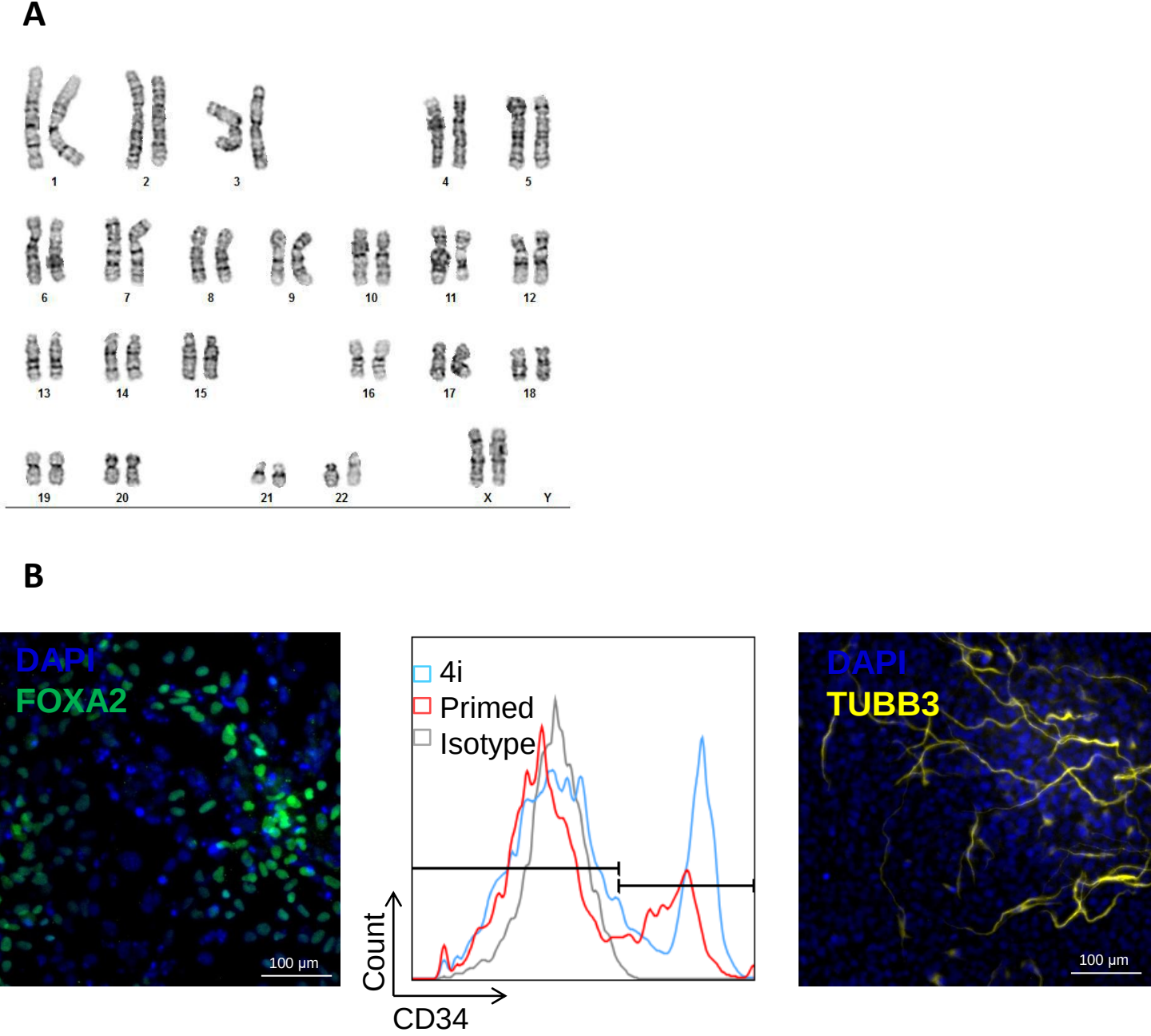


Figure S5: a) G-band karyotype of HES2 hPSC cultured for 5 passages in 5i in adherent conditions and 3 passages in 4i in suspension conditions. No karyotypic abnormalities are observed. b) Staining for endoderm (FOXA2), mesoderm (CD34), and ectoderm (TUBB3) markers in HES2 hPSC cultured for 5 passages in 5i in adherent conditions and 3 passages in 4i in suspension conditions.

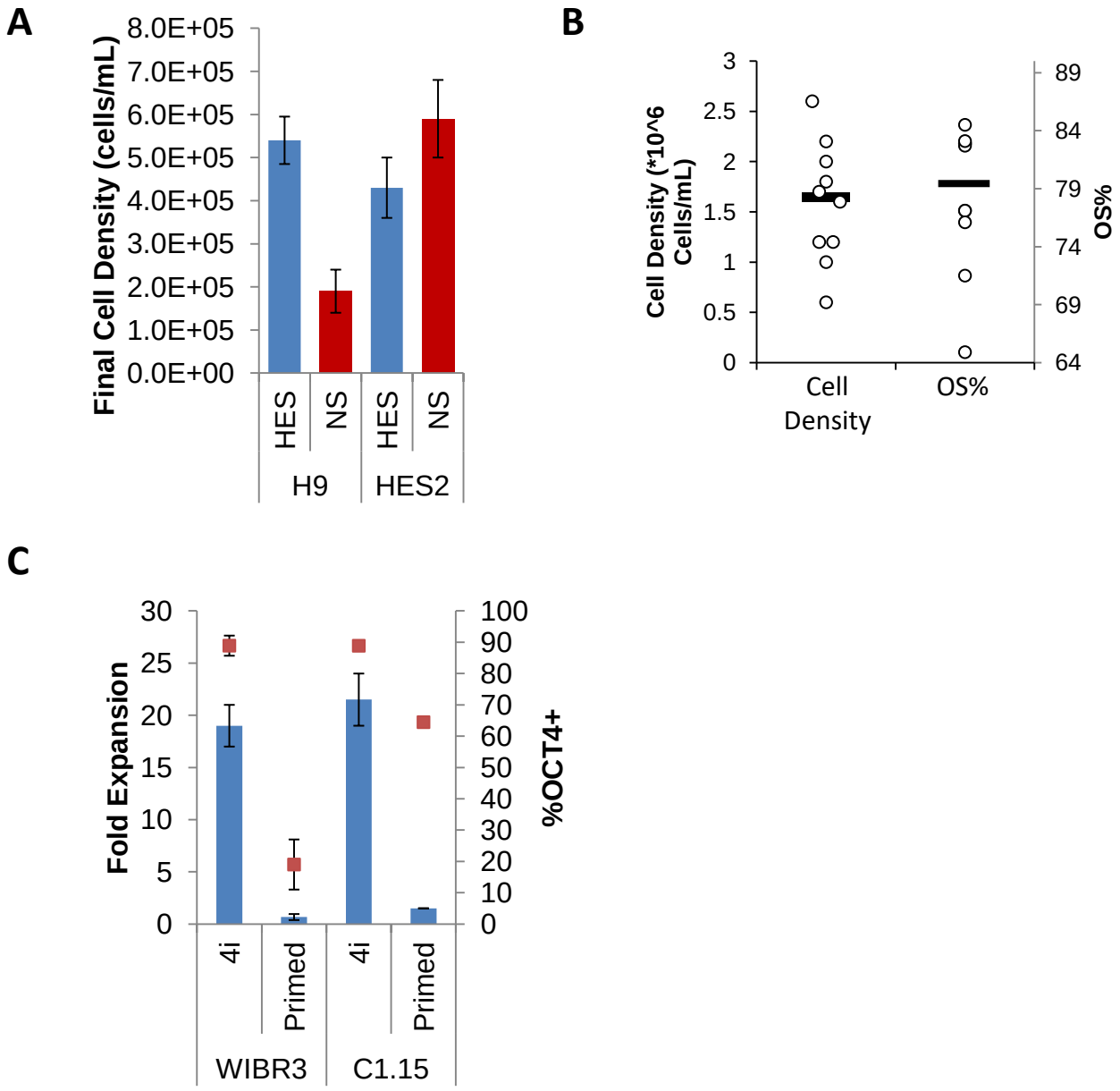


Figure S6: Final cell density and phenotype of primed hPSC. A) Final cell density reached after 6 days of suspension culture of primed HES2 and H9 hPSC in Serum Replacement (HES) medium and Nutristem (NS) medium. Error bars represent standard deviation. B) Final cell density and pluripotent phenotype (OS%: %OCT4+/SOX2+) of 4i-H9 in suspension culture. C) Fold expansion after 6 days in suspension culture and %OCT4-GFP+ cells after 8 days in suspension culture of primed and 4i- treated WIBR3 hESC and C1.15 iPSC. Primed cultures are expanded in HES medium.

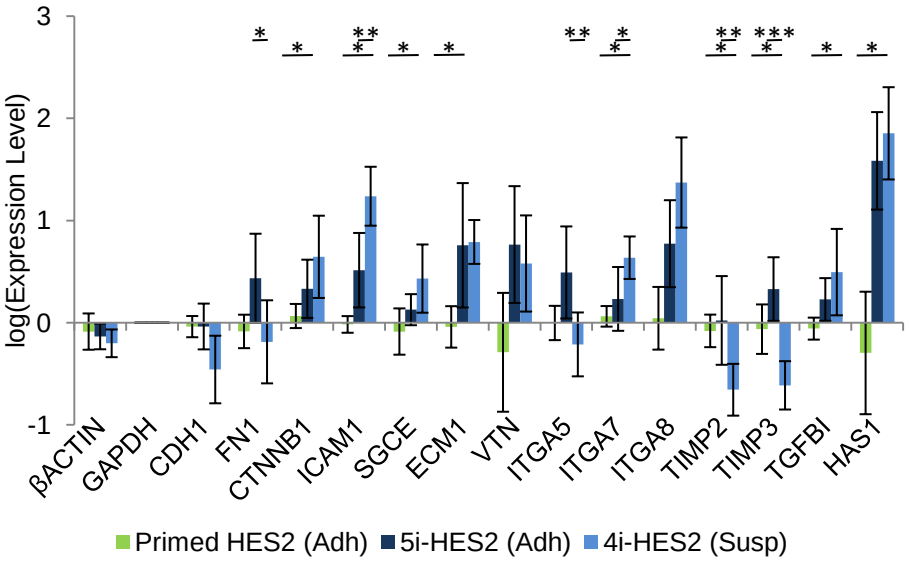


Figure S7: Suspension 4i-hPSC are Characterized by Distinct Suspension Adhesion-Related GeneExpression. A) Expression of select adhesion-related molecules in adherent primed hPSC, adherent 5i-hPSC, and suspension 4i-hPSC. Expression level is normalized to average primed HES2 GAPDH expression level. Significant differences exist between both adherent and suspension 5i-HES2 and primed conditions in select adhesion-related genes as noted. (Tukey's HSD Test, *, **, and *** indicate $p<0.05$, 0.01, 0.001 respectively, biological replicates: primed n=4, 5i-adherent n=9, 4i-suspension n=6. Error bars represent standard deviation.)

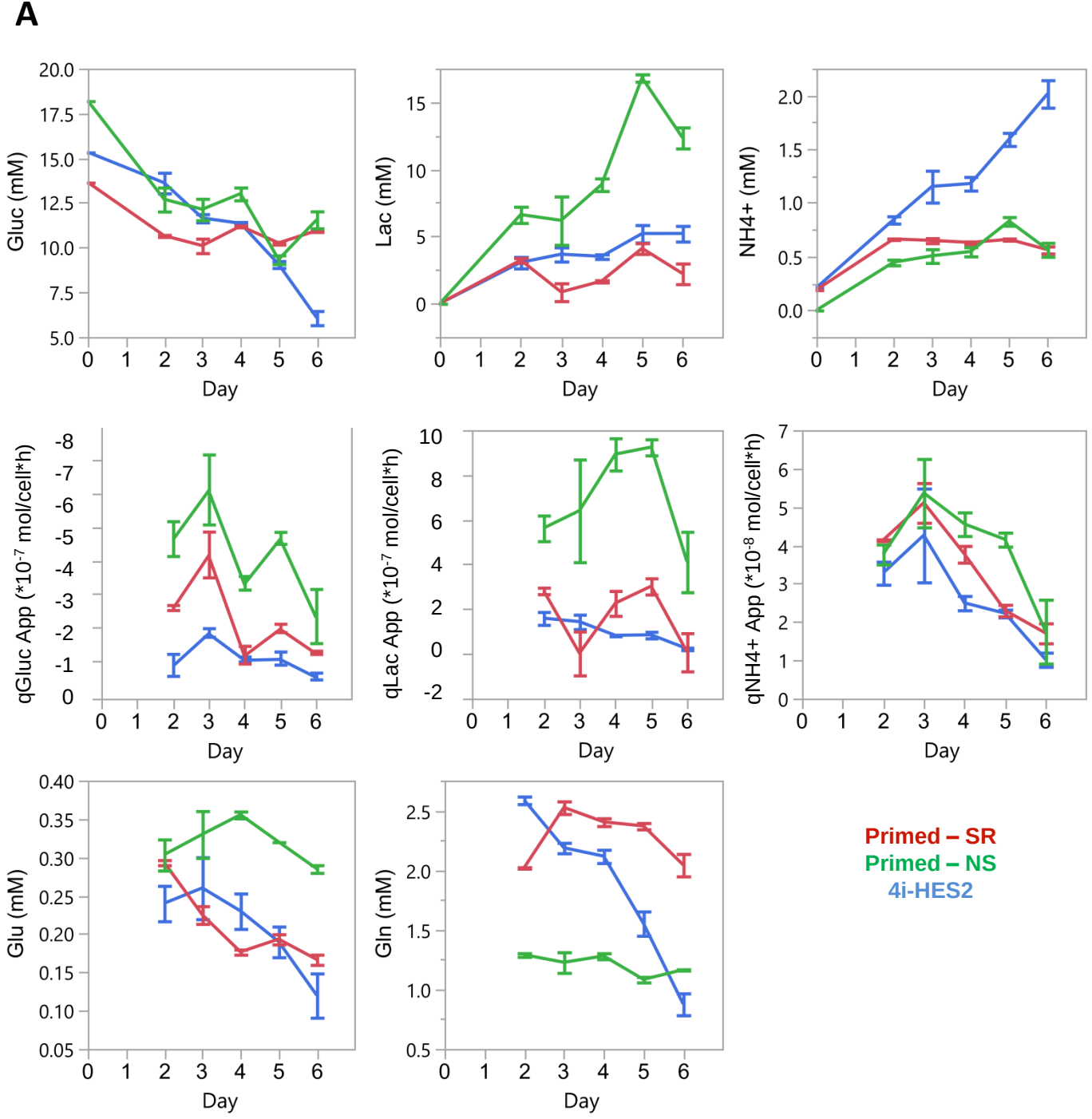
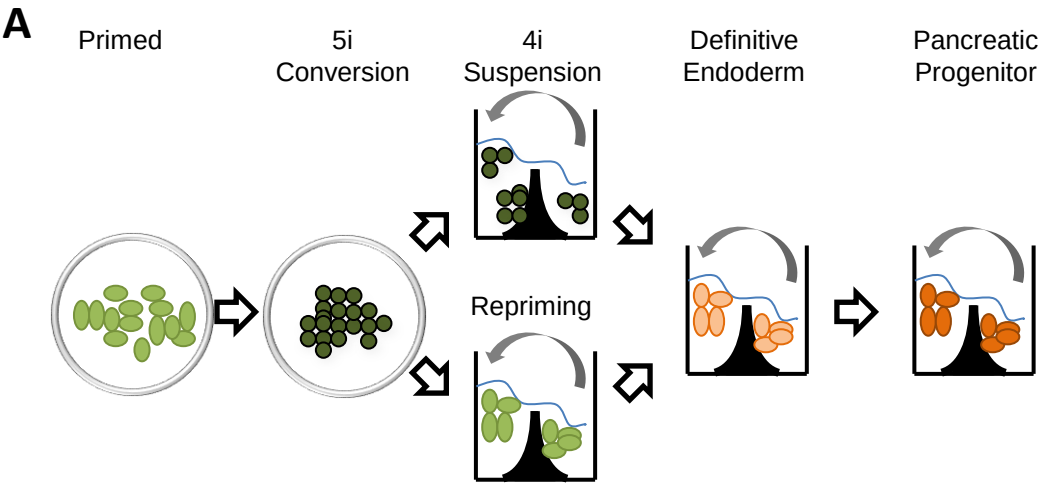


Figure S8: Level and metabolic rate of key metabolites in suspension expansion of hPSC. Primed hPSC are cultured in either NutriStem (NS) or Serum Replacement (SR) medium, compared to 4i-hPSC. 4i-hPSC are observed to have reduced specific uptake and secretion of glucose and lactate as well as rapidly depleting glutamine and glutamate levels. Error bars represent the standard deviation.



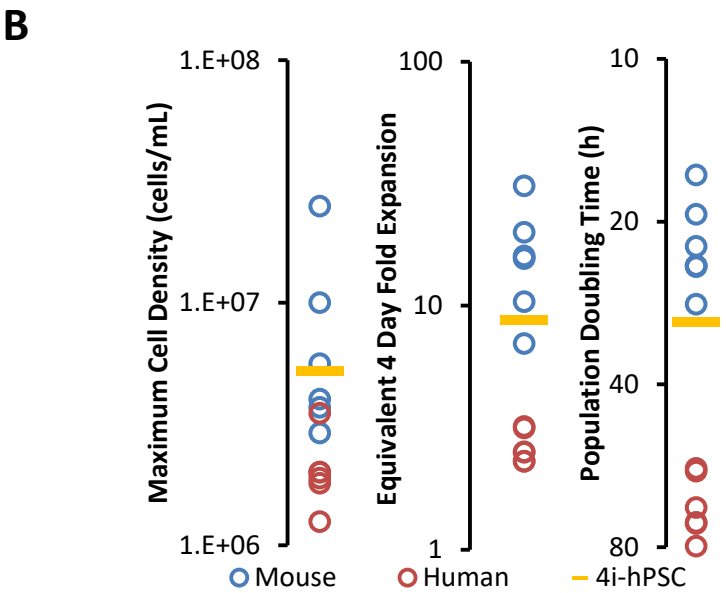


Figure S9: A) Differentiation of 5i-hPSC to pancreatic progenitors follows a 12-day directed differentiation protocol. Primed hPSC are converted to 4i-hPSC over several passages. 4i-hPSC are seeded into suspension and aggregate, followed by a 2 day re-priming stage. Definitive endoderm is induced between days 0-3 with Activin and CHIR followed by pancreatic progenitor specification at day 3-12. Days -2 to 0 are the "re-priming" stage in which 5i-hPSC aggregates are transferred to conventional primed hPSC medium. B) 4i-hPSC Suspension Expansion Yields Exceed Published hPSC Yields and Approach mPSC Yields. Maximum cell density, equivalent 4-day expansion, and suspension doubling times in the 4i-hPSC system are compared to literature published values (discussed in the introductory section) for bioreactor cell expansion of mouse and human PSC. Mouse (Blue) and human (Red) PSC are compared to the results obtained in the 4i-hPSC (yellow).

Table S1: Model of PD effect: A linear regression model indicates the significance of PD level on OCT4/SOX2 expression. Growth format and interaction effects were also significant

Regression Statistics	
Multiple R	0.707981
R Square	0.501238
Adjusted R Square	0.472463
Standard Error	18.48842
Observations	56

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	3	17862.93	5954.31	17.41935	5.88E-08
Residual	52	17774.72	341.8216		
Total	55	35637.65			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	51.6144	3.507931	14.71363	3.55E-20	44.57522	58.65358
PD	-27.0584	4.375158	-6.18455	9.83E-08	-35.8378	-18.279
Growth Format	-14.6832	4.960963	-2.95975	0.004628	-24.6381	-4.7283
PD*Format	18.8952	6.187408	3.053815	0.003558	6.479257	31.31114