HHS Public Access

Author manuscript

*Nat Methods*. Author manuscript; available in PMC 2013 December 01.

Published in final edited form as:

*Nat Methods*. 2013 June ; 10(6): 553–556. doi:10.1038/nmeth.2442.

# A simple tool to improve pluripotent stem cell differentiation

**Sundari Chetty**[1], **Felicia Walton Pagliuca**[1], **Christian Honore**[1], **Anastasie Kweudjeu**[2], **Alireza Rezania**[3], and **Douglas A. Melton**[1,4]

[1]Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

[2]CureMeta, 7 Drydock Avenue, Boston, MA, 02110, USA

[3]BetaLogics Venture, Janssen R & D LLC, Raritan, NJ, USA

[4]Howard Hughes Medical Institute, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

## Abstract

We develop a method to overcome previously documented restrictions on the differentiation propensities of pluripotent stem cells. Culturing pluripotent stem cells in dimethylsulfoxide (DMSO) activates the retinoblastoma protein, increases the proportion of cells in the early G1 phase of the cell cycle, and subsequently improves their competency for directed differentiation into multiple lineages in more than 25 stem cell lines. DMSO treatment also promotes terminal differentiation into functional derivatives.

The differentiation potential of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) varies substantially across cell lines. Certain cell lines have a higher capacity to differentiate into derivatives of some germ layers[1,2]. Based on differences in gene expression and DNA methylation profiles, a “lineage scorecard” has been established that predicts the differentiation potential of 32 hESC and hiPSC lines[2]. This has led to the view that particular cell lines need to be selected to achieve efficient differentiation to a lineage of choice.

We investigate the role of the cell cycle on differentiation potential and present an additional perspective. hESC and hiPSC lines have a cell cycle structure characterized by an abbreviated G1 gap phase and minimal checkpoint controls[3-6]. In early development, the embryonic cell cycle also has a truncated G1 phase during the period when rapid cell division occurs and decisions about fate and differentiation are held back[7-9]. Those studies suggest that the absence of an early G1 phase promotes self-renewal, and the presence of



*Correspondence to: Douglas A. Melton[1],,[4]dmelton@harvard.edu.

**AUTHOR CONTRIBUTIONS**

S.C. and D.A.M. conceived and designed the research, analyzed the data, and wrote the manuscript. S.C., F.W.P., C.H., A.K., and A.R. performed the experiments.

**COMPETING FINANCIAL INTERESTS**

A.R. is an employee of BetaLogics, a division of Janssen Research & Development, LLC, Raritan, NJ, USA.

this phase is associated with differentiation and cell fate changes. This led us to investigate whether the presence of an early G1 phase and its associated checkpoint controls are important for directed differentiation of pluripotent cell lines.

We show that culturing pluripotent stem cells in dimethylsulfoxide (DMSO) activates the retinoblastoma (Rb) protein (a regulator of the G1 restriction point)[7,9,10] and enhances the proportion of early G1 cells. We then show that DMSO overcomes previously reported restrictions on multilineage differentiation potential. In more than 25 different hESC and hiPSC lines, DMSO treatment increases the competency of pluripotent stem cells to respond to differentiation signals, enhances differentiation across all germ layers, and improves terminal differentiation into functional derivatives. This method permits differentiation of many cell lines toward a desired lineage and improves the prospects of using patient-specific iPSCs for disease modeling and autologous cell replacement therapy.

We began our analysis by investigating the hESC line HUES8. HUES8 has one of the highest propensities for differentiation to Sox17+ definitive endoderm cells[1,11], yet differentiation is not consistently high. By varying the initial plating density, we observed that the percent of cells that differentiate into definitive endoderm can range from 25% to 80% (Supplementary Fig. 1a-b), with the number of Sox17+ cells varying by as much as 7-fold (Supplementary Fig. 1c). Thus, cells are more responsive to differentiation signals if the differentiation protocol begins with cells plated at a high density. Since high density cultures are associated with increased contact-mediated growth inhibition and pluripotent stem cells have minimal sensitivity to contact inhibition[6], we hypothesized that promoting contact-mediated growth inhibition in hESCs might improve their response to differentiation signals.

In other tissue culture cell lines, culturing cells in DMSO can enhance contact inhibition and reversibly arrest cells in early G1 of the cell cycle[12-15]. Since responsiveness to differentiation signals is differentially regulated by density in HUES8 cultures, we assessed the effects of DMSO treatment on the differentiation potential of low and high density HUES8 cultures. HUES8 cultures were treated with 1% or 2% DMSO for 24 h and subsequently induced to differentiate into definitive endoderm. In low density cultures, this brief exposure to DMSO doubled responsiveness to differentiation signals (Supplementary Fig. 1d-e), increasing the percent of cells that become definitive endoderm from ~25% to 50%. DMSO treatment of high density HUES8 cultures resulted in high efficiencies comparable to control cultures (Supplementary Fig. 1e).

Next, we investigated whether DMSO treatment could improve the capacity to respond to differentiation signals in a cell line that has a low propensity to make definitive endoderm. Compared to HUES8, the HUES6 cell line is much less efficient at becoming endoderm even at high density[1,2] (Supplementary Fig. 1f). Treatment of HUES6 cells with 2% DMSO for 24 h prior to the onset of differentiation increased the percent of cells that became definitive endoderm from ~20% to 50% (Supplementary Fig. 1g). The H1 cell line is also predicted to have one of the lowest propensities towards the endodermal germ layer[2], yet DMSO treatment induced ~90%of H1 cells to become definitive endoderm (Supplementary Figs. 2c and 3a-b). In all three cell lines, cells that failed to differentiate retained expression

of pluripotent stem cell markers under control and DMSO conditions (Supplementary Fig. 2a-c).

Next, we assessed whether an initial DMSO treatment could improve terminal differentiation (Fig. 1a) in the low-propensity HUES6 line. A24 h DMSO treatment substantially improved HUES6 differentiation into Pdx1+ pancreatic progenitors (~60%) and terminally differentiated hormone expressing c-peptide+ cells (Fig. 1b). After the DMSO treatment, the terminal differentiation efficiency of the HUES6 line into c-peptide+ cells (~2-3%) is similar to the high-propensity HUES8 cell line[11] (Fig. 1c).

The HUES6 cell line is also predicted to be impaired in its ability to differentiate into the ectodermal and mesodermal lineages[1,2]. But an initial 24 h DMSO treatment significantly improved terminal differentiation into both lineages (Supplementary Figs. 4 and 5). Directed differentiation of HUES6 cells into the cardiomyocyte lineage following DMSO treatment generated contracting cardiomyocytes (Supplementary Videos 1-4; Supplementary Fig. 6), enabling functionality in a cell line previously considered incompetent.

We evaluated the long term effects of an initial DMSO treatment in two additional contexts. In low density cultures, HUES8 cells which had differentiated into definitive endoderm failed to differentiate further into Pdx1+ cells, and cell survival was substantially compromised by this stage (Fig. 1d-e). An initial 24 h DMSO treatment substantially improved long term differentiation potential in low density cultures, enhancing Pdx1 differentiation to 50%, comparable to high density cultures (Fig. 1d-f). DMSO treatment of low density cultures also promoted further terminal differentiation into endocrine cells (Supplementary Fig. 7).

Since pancreatic progenitor cells (i.e. Pdx1+ cells) may be a source for cell replacement therapy for diabetes[16-18], we assessed the effects of DMSO treatment on the Pdx1 differentiation potential in an iPS cell line derived from a type 1 diabetic subject (DiPS). While DiPS control cultures efficiently (~60%) differentiated towards the initial definitive endoderm stage, their ability to differentiate further to Pdx1 was significantly impaired even at high density. An initial 24 h DMSO treatment increased Pdx1 differentiation potential by 100-fold from 0.5% to 45% (Fig. 1d,g).

We show that DMSO has similar positive impacts across lineages in more than 25 other human pluripotent cell lines, including those used to generate the “lineage scorecard”[2]. Following directed differentiation (Fig. 2a), DMSO treatment enhanced differentiation potential into all three germ layers (Fig. 2b-c), both as a percentage of total cells (Supplementary Table 1) and in total yield (Supplementary Table 2). DMSO had particularly large effects in the poorest performing cell lines (Supplementary Fig. 8a). For instance, HUES1 is predicted to have the lowest differentiation potential for ectoderm as a result of suppressed expression of ectodermal-related genes[2]. Yet, DMSO treatment raised the ectodermal differentiation potential of HUES1 from 28% to 93% (Supplementary Fig. 8b-c and Supplementary Table 1).

Growth inhibition is closely associated with and regulated by a cell line’s cycling time. Since the average cycling times across many hESC and hiPSC lines commonly range from

~24-48 h[19,20], we assessed the DMSO effects on differentiation following a 24 h or 48 h treatment. Extending the DMSO treatment time to 48 h significantly improves differentiation rates above that observed following a 24 h DMSO treatment. The efficiencies across all germ layers have the capacity to reach differentiation potentials of 80-90% by optimizing the DMSO treatment (Supplementary Fig. 9a-b).

We also find that DMSO treatment has long term positive impacts on differentiation further down the endocrine lineage (Supplementary Fig. 10a). As in the DiPS 1000 cell line (Fig. 1), an initial 24 h DMSO treatment enhanced Pdx1 induction by nearly 15-fold and 5-fold on average in hESC and hiPSC lines, respectively (Supplementary Fig. 10b-c). Importantly, in cell lines that start with high efficiencies, DMSO significantly improved differentiation rates in later stages despite having modest impacts at the initial stage. Similarly, in the H1 cell line, the DMSO effects persisted to generate terminally differentiated endocrine cells (Supplementary Fig. 3c-d) at levels comparable to those reported in other high propensity cell lines[16,17].

We investigated the mechanisms by which DMSO treatment enhances the competency of pluripotent stem cells to respond to differentiation signals. We focused on the HUES6 line for this analysis because it exhibited the greatest improvements in differentiation potential. We investigated whether DMSO treatment regulates the distribution of pluripotent stem cells in the cell cycle as reported in other tissue culture systems[12-15]. Flow cytometry analysis of propidium iodide (PI) staining of HUES6 cells showed that a 24 h 2% DMSO treatment almost doubles the proportion of cells in G1 (Fig. 3a). To discriminate between cells in early and late G1, we also assessed the presence of the active hypophosphorylated retinoblastoma (Rb) protein, a marker of the early G1 phase of the cell cycle[9,21]. DMSO treatment was associated with a 4-fold increase in immunofluorescence reactivity of hypophosphorylated Rb (Fig. 3b) and substantially reduced levels of the phosphorylated and hyperphosphorylated forms of the Rb protein at several residues (ppRb; Fig. 3c-d). The accumulation of hypophosphorylated Rb indicates an overall enhancement in Rb activity and increased presence of the early G1 phase in DMSO treated cultures.

Live-cell imaging of HUES6 cells provided further evidence of changes in growth properties and cell contact during a 24 h DMSO treatment and subsequent release. DMSO treated cultures remain arrested until released from DMSO treatment and reached the same degree of confluence as control cultures (Supplementary Fig. 11). When cell contact was disrupted, DMSO no longer had an impact on improving differentiation potential (Fig. 3e) analogous to other cell types where loss of cell-to-cell contact is associated with inactivation of Rb and impaired differentiation[22]. Prolonged or permanent cell cycle arrest also inhibited DMSO-enhanced differentiation potential (Supplementary Fig. 12a-b), suggesting that opposing mechanisms may govern spontaneous extraembryonic differentiation[23,24] and directed differentiation of pluripotent stem cells.

We assessed whether similar changes in the cell cycle profile and Rb activity precede density-dependent improvements in differentiation. An increase in initial plating density significantly increased the proportion of cells in G1 in HUES8 (Fig. 3f) and reduced levels

of the phosphorylated and hyperphosphorylated forms of the Rb protein (Fig. 3g). Similar effects were also observed on the cell cycle of mouse ESCs following DMSO treatment.

In summary, the results presented here challenge the view that only some cell lines are suitable for differentiation into particular lineages. Culturing hESCs and hiPSCs in DMSO for a brief period (24-48 h) enables the use of multiple cell lines for differentiation into any lineage of choice with functional capabilities. Moreover, optimizing the duration of the DMSO treatment for a given cell line's cycling time further improves differentiation potential in all lines (see Supplementary Note for additional optimization).

While further work is needed to characterize the mechanism through which DMSO operates, our analysis suggests that DMSO likely improves differentiation by regulating Rb and the pluripotent cell cycle. Rb plays a key role in a variety of cellular processes, including tumorigenesis, senescence, apoptosis, DNA damage and repair, and genetic stability[9,10,25-27]. Regulating Rb activity using the tool described here could have applications in a variety of settings.

## Methods

### hESC/hiPSC maintenance conditions

All cell cultures were maintained at 37°C, 5% CO2. Human ESCs and iPSCs were grown on a monolayer of irradiated CF1-mouse embryonic fibroblasts (MEFs) (GlobalStem) in hESC media consisting of KO-DMEM (Invitrogen), 10% KOSR (Invitrogen), 1% glutamax, nonessential amino acids, penicillin/streptomycin, 0.1% β-mercaptoethanol supplemented with 10ng/ml bFGF (Invitrogen). Media was changed every 24h and lines were passaged using TrypLE (Invitrogen). Prior to differentiation experiments, cells were expanded for at least two passages on matrigel (BD Biosciences) in MEF-conditioned media (R&D Systems) with 20ng/mL bFGF supplemented with 10μM of the ROCK inhibitor Y-27632[31].

HUES and hiPS cell lines were obtained from the Human Embryonic Stem Cell Facility and the iPS Core Facility of the Harvard Stem Cell Institute. HUES cell lines were previously derived and characterized by [19] and [1], respectively. hiPS and DiPS cell lines were previously derived and characterized by [32] and [33], respectively. Other hiPS cell lines reported in the study were derived via retroviral or sendai viral reprogramming with 3 (Oct4, Sox2, Klf4) or 4 (Oct4, Sox2, Klf4, and c-Myc) factors: 1015-3F B (3-factor retroviral), 1013-4FA (4-factor retroviral), iPS Sev A and iPS Sev B (4-factor sendai viral). The hES cell lines BGO1V/hOG (Gibco), H1, H9, and H14 (WiCell) were also included in the study.

### Differentiation protocols

For all differentiation studies, hESC/iPSCs were plated onto wells coated with growth factor reduced matrigel (BD Biosciences) in MEF-conditioned media with 20ng/ml bFGF and 10μM of the ROCK inhibitor Y-27632. Plating densities were as follows:0.25 million/well of a 6-well plate (low density), 0.5 million/well of a 6-well plate (medium density), 1 million/well of a 6-well plate (high density) or 100,000/well of a 96-well plate (high density). Multilineage differentiation potential across cell lines was assessed at high density. 24h or 48h prior to the onset of differentiation, cells were cultured in MEF-conditioned

media with 20ng/ml bFGF and 1% or 2% DMSO (with two DMSO treatments for a 48h treatment time). This medium was removed and replaced with each of the following medium at the start of each differentiation, with replacement of media every day in all protocols.

**a.** *Ectoderm*: Cells were cultured in KO-DMEM (Invitrogen) medium, containing 10% knockout serum replacement (Invitrogen), supplemented with Noggin (500 ng/ml; R&D Systems) + SB431542 (10 μM; Tocris) for 4d (hESCs) or 2d (hiPSCs), time points corresponding to the peak expression of Sox1[34].

**b.** *Mesoderm (4d induction)*: Cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant Activin A (100 ng/ml; R&D Systems) for 1d followed by human recombinant BMP4 (10 ng/ml; R&D Systems) for 3 days. *Mesoderm (1d induction)*: Since repeated BMP4 treatment can also promote differentiation into non-mesodermal fates, another second mesodermal induction protocol known to reduce differentiation into alternative fates[28] was also assessed: cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant Activin A (100ng/ml) + Wnt3a (20ng/ml; R&D Systems) for 24h.

**c.** *Endoderm*: Cells were cultured in MCDB-131 medium (Invitrogen) supplemented with $NaHCO_3$ (2.5g/l), 1% glutamax, glucose (5.5mM), 0.1% FAF-BSA (Proliant/ Lampire), and ITS:X (1:50,000; Invitrogen) and treated with Wnt3a (20ng/ml) +AA (100ng/ml) for 1d and AA (100ng/ml) for 3d.

**d.** *Terminal endocrine differentiation*: Cells were cultured in MCDB-131 medium supplemented with $NaHCO_3$ (2.5g/l), 1% glutamax, glucose (5.5mM), 0.1% FAF-BSA, and ITS:X (1:50,000), and treated with Wnt3a (20ng/ml) + AA (100ng/ml) for 1d, AA (100ng/ml) for 3d, and FGF7 (50ng/ml; Peprotech) for 2d. For the following 13d, cells were cultured in MCDB-131 medium supplemented with $NaHCO_3$ (2.5g/l), 1% glutamax, glucose (8mM), 2% FAF-BSA, and ITS:X (1:200) and treated for 4d with FGF7 (50ng/ml) +Noggin (100ng/ml) +retinoic acid (2μM; Sigma) +Sant-1 (0.25μM; Sigma) +AA (20ng/ml); 3d with Sant-1 (0.25μM) + PdBu (200nM; EMD) +Noggin (100ng/ml); and 4-6d with Noggin (100ng/ml) + Alk5 inhibitor (1μM; Axxora).

**e.** *Terminal mesodermal differentiation*: Cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant Activin A (100 ng/ml; R&D Systems) for 1d followed by human recombinant BMP4 (10 ng/ml; R&D Systems) for 3 days. Medium was then replaced every 2 days for another 8d with RPMI-B27 without supplementary cytokines [28]. Cells were differentiated into cardiomyocytes following temporal modulation of Wnt signaling[35].

**f.** *Terminal ectodermal differentiation*: Cells were cultured in KO-DMEM (Invitrogen) medium, containing 10% knockout serum replacement (Invitrogen), supplemented with Noggin (500 ng/ml; R&D Systems) + SB431542 (10 μM; Tocris) for 4d, and allowed to differentiate in Noggin alone for the remaining 8d as in[34].

### Immunocytochemistry and quantification

Cells were rinsed in PBS and fixed in 4% paraformaldehyde (PFA; Sigma) for 30 minutes. Following rinses, cells were blocked for 1h at room temperature in 5% donkey serum (Jackson Immunoresearch), 0.3% triton, in PBS. All primary antibody incubations were done overnight in blocking solution at a 1:500 dilution unless otherwise noted: Sox1 (ectoderm; R&D systems), Brachyury (mesoderm; R&D systems), Sox17 (definitive endoderm; R&D systems), Pdx1 (pancreatic progenitor; R&D systems), c-peptide (endocrine cell; Cell signaling), glucagon (endocrine cell; Cell signaling), nestin (neural; R&D systems), Tuj1 (neural; Covance), Troponin C and Troponin T (mesodermal and cardiomyocyte; Abcam), Nkx2.5 (Cardiac specific homeobox protein; R&D systems), Phospho-Rb-Ser780 (Cell Signaling), and hypophosphorylated Rb (1:50 dilution; BD Pharmingen). Cells were rinsed the next day followed by secondary antibody incubation for 1h at room temperature at a 1:500 dilution. Secondary antibodies (Invitrogen) conjugated to AlexaFluor 488 or 594 were used to visualize primary antibodies. Following PBS rinses, all nuclei were visualized by staining with Hoechst 33342 (1:1000, Molecular Probes).

**Quantification—**In each of the differentiation experiments, 2-4 wells from the same passage were assessed for differentiation potential. Using the Cellomics high content screening system, thirty 10x fields per well were acquired and quantified. Cells labeled by antibody staining and total cell number (based on DAPI nuclei staining) were quantified to obtain percentages of target cell types.

### Flow Cytometry

hESC-derived cells were released into single-cell suspension by incubation in TrypLE Express (Invitrogen) at 37°C for 3-5 minutes. Cells were then washed twice in staining buffer (PBS containing 0.2% BSA) (BD Biosciences). For surface marker staining, $1\times10^5$ to $1\times10^6$ cells were re-suspended in 100 µl blocking buffer (0.5% human gamma-globulin diluted 1:4 in staining buffer). Directly conjugated primary antibodies (CD184/CXCR4 APC (Allophycocyanin) and CD9 PE; BD Biosciences)were added to the cells at a final dilution of 1:20 and incubated for 30 min at 4°C. Stained cells were washed twice in BD staining buffer, re-suspended in 200 µl staining buffer, followed by incubation in 15 µl of 7AAD for live/dead discrimination prior to analysis on the BD FACS Canto II.

For intracellular antibody staining, cells were first incubated with Green Fluorescent LIVE/DEAD cell dye (Invitrogen) for 20 min at 4°C to allow for live/dead cell discrimination during analysis, followed by a single wash in cold PBS. Cells were fixed in 250 µl of Cytofix/Cytoperm Buffer (BD) for 20 min at 4°C followed by two washes in BD Perm/Wash Buffer Solution (BD). Cells were re-suspended in 100 µl staining/blocking solution consisting of Permwash buffer with 2% normal goat serum (or appropriate species of the secondary antibody). Cells were then incubated for 30 min at 4°C with primary antibodies at empirically pre-determined dilutions followed by two washes in Perm/Wash buffer. Lastly, cells were incubated with the appropriate secondary antibodies for 30 min at 4°C followed by two washes prior to analysis on the BD FACS Canto II. The following concentrations of primary antibodies were used: goat SOX17 (1:20; R&D Systems), HNF3beta (1:20; BD Biosciences), mouse anti-NKX6.1 (1:50; DSHB, University of Iowa);rabbit anti-

synaptophysin (1:100; Dako A0010; Carpinteria, CA), Islet1 (1:50, Abcam). For secondary antibodies, goat anti-mouse Alexa 647 (1:500; Invitrogen), goat anti-rabbit PE (1:200; Invitrogen) or donkey anti-goat Alexa 647 (1:800; Invitrogen) were added and incubated for 30 min at 4°C followed by a final wash in Perm Wash buffer. Cells were analyzed on BD FACS Canto II using the BD FACS Diva Software with at least 30,000 events being acquired.

### Quantitative RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen; Valencia, CA) and reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. cDNA (100-120 ng) was amplified by PCR using Taqman Universal Master Mix and Taqman Gene Expression Assays which were pre-loaded onto custom Taqman Arrays (Applied Biosystems). Data were analyzed using Sequence Detection Software (Applied Biosystems) and normalized to undifferentiated hESCs using the ΔΔCt method. A list of primers (Applied Biosystems) is provided in[36].

### Live-cell imaging

Phase-contrast imaging of cultures undergoing a 24h DMSO treatment followed by a 24h release in hESC maintenance media was acquired using the IncuCyte live-cell imaging system.

### Cell cycle analysis

Cells were trypsinized and fixed in 100% EtOH for 30 minutes on ice, treated with RNaseA (100 μg/ml, Qiagen) and stained with propidium iodide (PI; 50 μg/ml, Invitrogen) in 0.1% triton for at least 30 min. Cells were assessed on the BD Biosystem LSRII FACS flow cytometer and analyzed using the FlowJo software.

**Western blot analysis—**Cells were lysed using the RIPA Lysis Buffer (Santa Cruz). Proteins were separated by 7.5 % SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% skim milk in 0.1% triton for 1 hour at room temperature and then incubated with the following primary antibodies overnight at 4°C : G3-245 (ppRb, 1:1000, BD Pharmingen), Phospho-Rb-Ser780 (1:200, Cell Signaling), Phospho-Rb-Ser795 (1:200, Cell Signaling), Phospho-Rb-Ser807/811 (1:200, Cell Signaling), 4H1 (total Rb, 1:1000, Cell Signaling), or GAPDH (1:5000, Abcam) as the loading control. Following washes, the membranes were incubated with secondary antibodies for 1h at room temperature: anti-mouse HRP (1:1000, Cell Signaling) or anti-rabbit HRP (1:1000, Cell Signaling), and then incubated in Chemiluminescent HRP substrate (Millipore) for signal detection and development.

### Statistical analysis

Means and SEMs were determined for the above variables. For statistical comparisons, these values were subjected to unpaired or paired (across multiple cell lines) two-tailed Students t-tests. P-values ≤ 0.05 were considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank E. Scadden for her excellent technical support, R. Maehr, P. Makhijani, and J. Millman of Harvard University for providing many of the hiPSC lines, K. Lam for assistance with the Cellomics high content screening system, and A. Meissner, K. Eggan, D. Cohen, R. Maehr, J. Rivera-Feliciano, B. Blum, J. Sneddon, and Q. Peterson for their comments on the manuscript. This work was supported by the Harvard Stem Cell Institute, Howard Hughes Medical Institute, the Leona M. and Harry B. Helmsley Charitable Trust, NovoNordisk, and the NIH (1R24DK092758 and 1U01HL10040804).

## References

1. Osafune K, et al. Marked differences in differentiation propensity among human embryonic stem cell lines. Nature Biotechnology. 2008; 26:313–315.
2. Bock C, et al. Reference Maps of Human ES and iPS Cell Variation Enable High-Throughput Characterization of Pluripotent Cell Lines. Cell. 2011; 144:439–452. [PubMed: 21295703]
3. Smith AG. Embryo-derived stem cells: Of mice and men. Annual Review of Cell & Developmental Biology. 2001; 17:435–62.
4. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. Nature Reviews Genetics. 2008; 9:115–128.
5. Conklin JF, Sage J. Keeping an eye on retinoblastoma control of human embryonic stem cells. Journal of Cellular Biochemistry. 2009; 108:1023–1030. [PubMed: 19760644]
6. Ben-David U, Benvenisty N. The tumorigenicity of human embryonic and induced pluripotent stem cells. Nature Reviews Cancer. 2011; 11:268–277. [PubMed: 21390058]
7. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science. 1989; 246:629–34. [PubMed: 2683079]
8. Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic cell cycle. Nature. 1989; 331:275–280. [PubMed: 2566917]
9. Pardee AB. G1 events and regulation of cell proliferation. Science. 1989; 246:603–608. [PubMed: 2683075]
10. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell. 1995; 81:323–330. [PubMed: 7736585]
11. Chen S, et al. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nature Chemical Biology. 2009; 5:258–265. [PubMed: 19287398]
12. Sawai M, Takase K, Teraoka H, Tsukada K. Reversible G1 arrest in the cell cycle of human lymphoid cell lines by dimethyl sulfoxide. Experimental Cell Research. 1990; 187:4–10. [PubMed: 2298260]
13. Teraoka H, Mikoshiba M, Takase K, Yamamoto K, Tsukada K. Reversible G1 Arrest Induced by Dimethyl Sulfoxide in Human Lymphoid Cell Lines: Dimethyl Sulfoxide Inhibits IL-6-Induced Differentiation of SKW6-CL4 into IgM-Secreting Plasma Cells. Experimental Cell Research. 1996; 222:218–224. [PubMed: 8549666]
14. Ponzio G, et al. Early G1 growth arrest of hybridoma B cells by DMSO involves cyclin D2 inhibition and p21[CIP1] induction. Oncogene. 1998; 17:1159–66. [PubMed: 9764826]
15. Fiore M, Degrassi F. Dimethyl Sulfoxide Restores Contact Inhibition-Induced Growth Arrest and Inhibits Cell Density-Dependent Apoptosis in Hamster Cells. Experimental Cell Research. 1999; 251:102–110. [PubMed: 10438575]
16. D'Amour KA, et al. Production of pancreatic hormone expressing endocrine cells from human embryonic stem cells. Nature Biotechnology. 2006; 24:1392–1401.
17. Kroon E, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nature Biotechnology. 2008; 26:443–452.

18. Borowiak M, Melton DA. How to make β cells? Current Opinion in Cell Biology. 2009; 21:727–732. [PubMed: 19781928]
19. Cowan CA, et al. Derivation of Embryonic Stem-Cell Lines from Human Blastocysts. New England Journal of Medicine. 2004; 350:1353–1356. [PubMed: 14999088]
20. Takahashi K, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell. 2007; 131:861–872. [PubMed: 18035408]
21. Chen PL, Scully P, Shew JY, Wang JY, Lee WH. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell. 1989; 58:1193–1198. [PubMed: 2673546]
22. Sosa-García B, et al. A Role for the Retinoblastoma Protein As a Regulator of Mouse Osteoblast Cell Adhesion: Implications for Osteogenesis and Osteosarcoma Formation. PLoS ONE. 2010; 5:1–16.
23. Ruiz S, et al. A High Proliferation Rate Is Required for Cell Reprogramming and Maintenance of Human Embryonic Stem Cell Identity. Current Biology. 2011; 21:45–52. [PubMed: 21167714]
24. Sela Y, Molotski N, Golan S, Itskovitz-Eldor J, Soen Y. Human Embryonic Stem Cells Exhibit Increased Propensity to Differentiate During the G1 Phase Prior to Phosphorylation of Retinoblastoma Protein. Stem Cells. 2012; 30:1097–1108. [PubMed: 22415928]
25. Jacks T, et al. Effects of an Rb mutation in the mouse. Nature. 1992; 359:295–300. [PubMed: 1406933]
26. Slack RS, et al. Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. J Cell Biol. 1995; 129:779–788. [PubMed: 7730411]
27. Nguyen DX, Baglia LA, Huang S-M, Baker CM, McCance DJ. Acetylation regulates the differentiation-specific functions of the retinoblastoma protein. EMBO Journal. 2004; 23:1609–1618. [PubMed: 15044952]
28. Zhang P, et al. Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. Blood. 2008; 111:1933–1941. [PubMed: 18042803]
29. Kattman SJ, et al. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell. 2011; 8:228–240. [PubMed: 21295278]
30. Nostro MC, et al. Stage-specific signaling through TGFβ family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development. 2011; 138:861–871. [PubMed: 21270052]
31. Watanabe K, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nature Biotechnology. 2007; 25:681–686.
32. Boulting GL, et al. A functionally characterized test set of human induced pluripotent stem cells. Nature Biotechnology. 2011; 29:279–286.
33. Maehr R, et al. Generation of pluripotent stem cells from patients with type 1 diabetes. PNAS. 2009; 106:15768–15773. [PubMed: 19720998]
34. Chambers SM, et al. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature Biotechnology. 2009; 27:275–80.
35. Lian X, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. PNAS. 2012; 109:1848–1857.
36. Rezania A, et al. Production of functional glucagon-secreting α-cells from human embryonic stem cells. Diabetes. 2011; 60:239–247. [PubMed: 20971966]

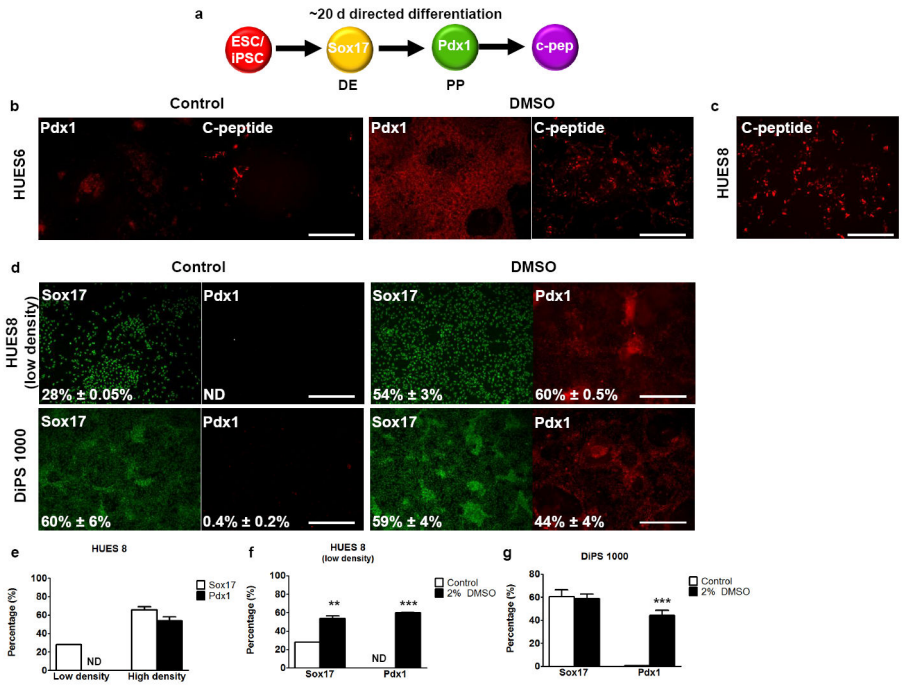


**Figure 1. DMSO treatment at the pluripotent stage enhances differentiation potential at subsequent stages of stepwise differentiation in hES and hiPS cell lines**

**(a)** Schematic of stepwise differentiation into pancreatic (Pdx1+) progenitor cells and terminally differentiated c-peptide (c-pep+) cells following a 20 d directed differentiation protocol. **(b)** In the low propensity HUES6 cell line, treatment with DMSO prior to the 20 d directed differentiation protocol significantly improves differentiation into Pdx1+ pancreatic progenitors (~60%) and further promotes terminal differentiation of c-peptide+ cells at levels similar to the high propensity **(c)** HUES8 cell line. **(d, e)** In low density HUES8 cultures, HUES8 cells that differentiate into Sox17+ definitive endoderm fail to differentiate further to Pdx1+ cells, and cell survival is substantially compromised. At high densities, HUES8 cultures efficiently (~60%) differentiate into Sox17+ definitive endoderm and Pdx1+ pancreatic progenitors. **(d, f)** In low density HUES8 cultures, a 24 h DMSO treatment prior to the start of directed differentiation enhances Pdx1 differentiation up to 50%, significantly improving long term differentiation potential. **(d, g)** In an iPS line derived from a diabetic patient (DiPS 1000), about 60% of the cells differentiate into Sox17+ definitive endoderm in control and DMSO treated cultures. Subsequent differentiation into Pdx1+ cells, however, is significantly reduced to <1% in control cultures, whereas Pdx1 differentiation is ~45% in DMSO treated cultures. DE, definitive endoderm, PP, pancreatic progenitor, ND, not detected; Error bars represent SEM of 2-4 biological replicates; Scale bar, 200μM; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

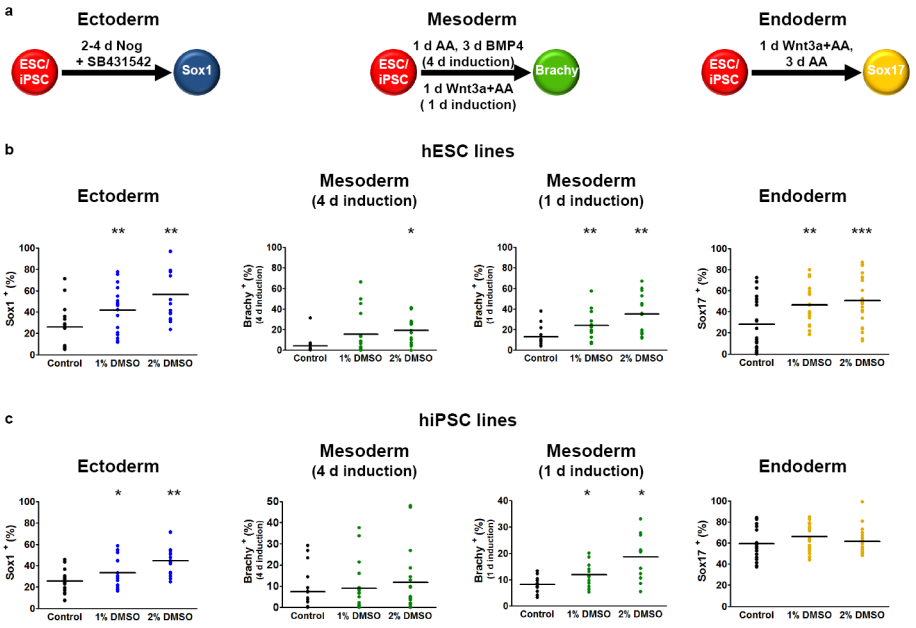


**Figure 2. DMSO treatment improves differentiation into all germ layers across multiple human ES and iPS cell lines**

**(a)** Schematic of directed differentiation protocols used to assess differentiation potential of multiple human ESC and iPSC lines into the ectodermal, mesodermal, and endodermal germ layers following a 24 h treatment with 1% or 2% DMSO. The percent of cells differentiating into Sox1 (ectoderm), Brachyury (mesoderm), and Sox17 (endoderm) was quantified by automated image analysis. Since the mesodermal BMP4 protocol is known to also promote trophoblast and extra-embryonic differentiation[28] (also a by-product in our BMP4-induced differentiations), a second mesodermal induction protocol was also used to assess mesodermal potential in a subset of the lines. **(b)** A 24 h DMSO treatment prior to the onset of differentiation significantly enhances the propensity for differentiation across all hESC lines into all germ layers. On average, control lines differentiate into each of the germ layers at an efficiency of approximately 5-25%. DMSO treatment increases these levels up to 20-50%, with fold changes ranging from 2 -20 fold. **(c)** The average effect of DMSO treatment on all hiPSC lines enhances ectodermal and mesodermal differentiation potential by 2-3 fold. A 60% endodermal differentiation potential in the average iPSC line is not significantly improved by a 24 h DMSO treatment. Each point corresponds to an individual well; 2-4 biological replicates for each cell line. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

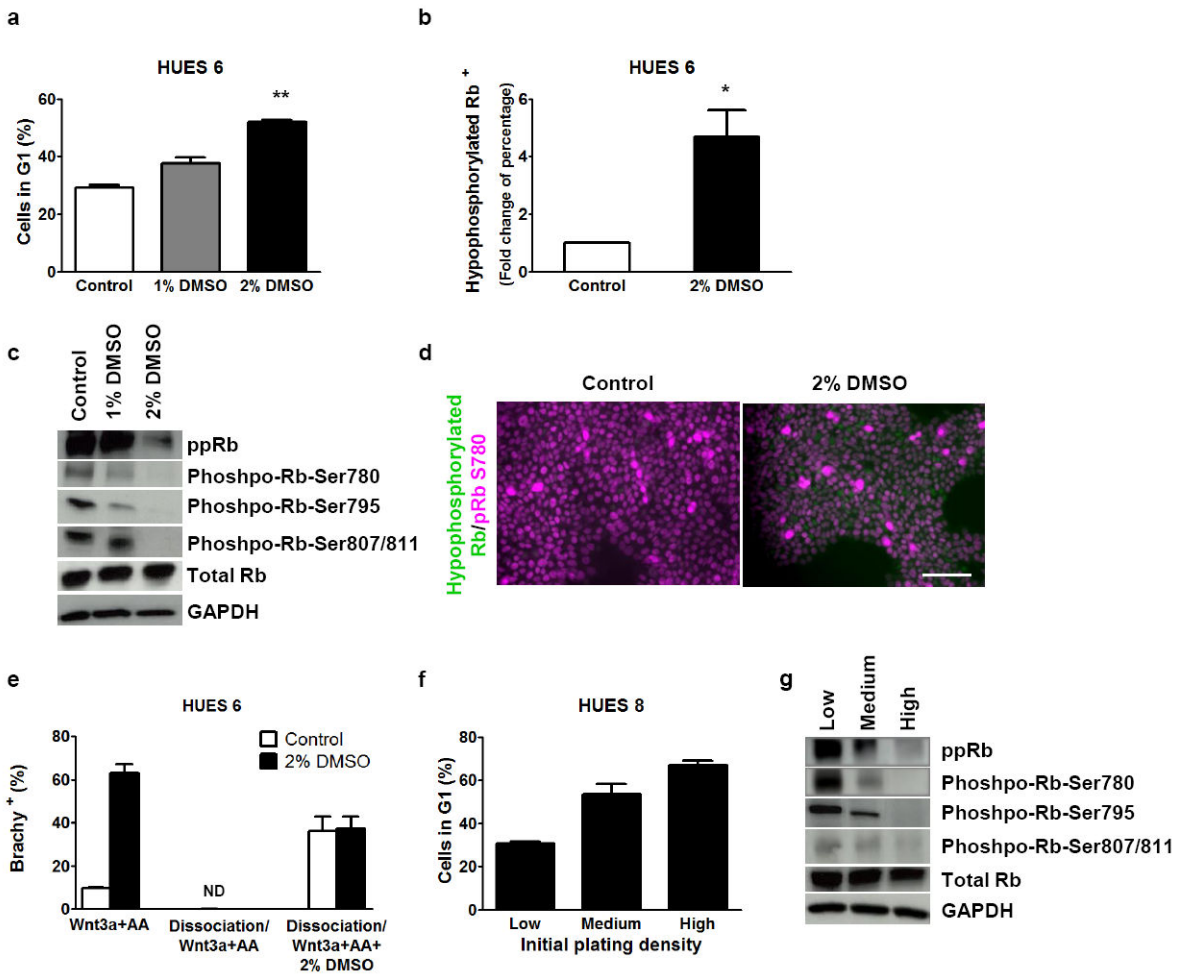


**Figure 3. DMSO-enhanced differentiation potential is preceded by Rb activation and an increased proportion of early G1 cells**

**(a)** Prior to the onset of differentiation, cell cycle analysis (propidium iodide staining)of HUES6 cells shows that a 24 h treatment with 2% DMSO nearly doubles the proportion of cells in the G1 phase of the cell cycle. **(b)** A 24 h 2% DMSO treatment increases the proportion of cells expressing the active hypophosphorylated Rb by approximately 4-fold, indicating an increased proportion of cells in early G1. **(c, d)** DMSO treatment concomitantly reduces protein levels of phosphorylated and hyperphosphorylated Rb (ppRB). Further assessment of phospho-specific Rb antibodies shows that 2% DMSO reduces Rb phosphorylation at several residues (Ser780, Ser795, and Ser807/811). **(e)** A 60% mesodermal differentiation into Brachy+ cells following DMSO treatment of HUES6 cells is inhibited when cells are dissociated, re-plated, and induced with Wnt3a+AA. Dissociation and induction with Wnt3a+AA in the presence of DMSO enhances differentiation potential to ~40%. **(f)** Prior to the onset of differentiation in HUES8 cells, cell cycle analysis shows that an increase in cell density enhances the proportion of cells in the G1 phase from 30% to 70%. **(g)** High density HUES8 cultures are also associated with reduced levels of the phosphorylated and hyperphosphorylated forms of the Rb protein (ppRb). Rb phosphorylation at several residues (Ser780, Ser795, and Ser807/811) is reduced

with an increase in cell density, indicative of enhanced Rb activity and the presence of an early G1 phase. ND, not detected; Error bars represent SEM of 2-4 biological replicates. Scale bar, 200μM; *$p \leq 0.05$, **$p \leq 0.01$.