We describe a method to help overcome restrictions on the differentiation propensities of human 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, in more than 25 embryonic and induced pluripotent stem cell lines, improves directed differentiation into multiple lineages. DMSO treatment also improves differentiation into terminal cell types in several cell lines.

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 layers1,2. Based on differences in gene expression and DNA methylation profiles, a ‘lineage scorecard’ has been described that predicts the differentiation potential of 32 hESC and hiPSC lines3. This has led to the view that particular cell lines need to be selected to achieve efficient differentiation to a lineage of choice.

We investigated the role of the cell cycle on differentiation potential and present a new perspective, challenging the view that only some human pluripotent stem cell lines are suitable for differentiation into particular lineages. hESC and hiPSC lines have a cell-cycle structure characterized by an abbreviated G1 gap phase and minimal checkpoint controls5–6. Similarly, in early development in vivo, 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 back7–10. These findings suggest that the absence of an early G1 phase is associated with self-renewal and that the presence of 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 began our analysis by investigating the hESC line HUES8. HUES8 has one of the highest propensities for differentiation to Sox17+ definitive endoderm cells11,12, yet its differentiation is not consistently high. By varying the initial plating density, we observed that the percentage of cells that differentiate into definitive endoderm (Fig. 1a) can range from 25% to 80% (Fig. 1b and Supplementary Fig. 1a,b), with the number of Sox17+ cells varying by as much as sevenfold (Supplementary Fig. 1c). Cells were more responsive to differentiation signals if the differentiation protocol began with cells plated at a high density. We hypothesized that promoting contact-mediated growth inhibition in hESCs, as in high-density cultures, might improve their response to differentiation signals.

DMSO treatment has been shown to enhance contact inhibition and reversibly arrest cells in early G1 in other cultured cell lines13–15. In addition, pleiotropic effects of DMSO, including on the spontaneous differentiation of embryoid bodies, have been noted in various cultured cell types16,17.

We assessed the effects of DMSO treatment on the differentiation potential of low- and high-density HUES8 cultures. The cultures were treated with 1% or 2% DMSO for 24 h and subsequently induced to differentiate into definitive endoderm. In low-density cultures, exposure to DMSO doubled responsiveness to differentiation signals (Fig. 1b and Supplementary Fig. 1d), increasing the percentage of Sox17+ cells from ~25% to 50%. DMSO treatment of high-density HUES8 cultures resulted in high efficiencies comparable to those of control cultures (Fig. 1b).

Next we investigated the effect of DMSO treatment on HUES6, a cell line with low propensity to make definitive endoderm even at high density12 (Fig. 1c). Treatment of HUES6 cells with 2% DMSO for 24 h before the onset of differentiation increased Sox17+ cells from ~20% to 50% (Fig. 1d and Supplementary Fig. 1e). The H1 cell line is also predicted to have one of the lowest propensities to differentiate toward the endodermal germ layer12; DMSO treatment induced ~90% of H1 cells to become Sox17+ definitive endoderm (Supplementary Figs. 2c and 3a,b). In all three lines, cells that failed to differentiate retained expression of pluripotency markers under control and DMSO conditions (Supplementary Fig. 2).

Next we assessed whether an initial DMSO treatment could improve terminal differentiation in HUES6. A 24-h DMSO treatment substantially improved HUES6 differentiation into Pdx1+ pancreatic progenitors (~60%, Fig. 1d) and terminally differentiated hormone-expressing C-peptide+ cells to levels similar to those seen for the high-propensity HUES8 cell line11 (Supplementary Fig. 1f). Other terminal cell types induced by this protocol were not assessed.

HUES6 is also predicted to be impaired in its ability to differentiate into the ectodermal (neural) and mesodermal lineages12. An initial 24-h DMSO treatment significantly improved terminal differentiation potential of 32 hESC and hiPSC lines2. This has led to a ‘lineage scorecard’ that predicts the differences in gene expression and DNA methylation profiles, cell types in several cell lines.

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Differentiation into both lineages (P ≤ 0.05; Supplementary Figs. 4 and 5), as assessed by the expression of nestin and Tuj1 for the neural lineage and of Nkx2.5, troponin C and troponin T for the mesodermal lineage. DMSO treatment permitted differentiation of HUES6 cells into contracting cardiomyocytes (Supplementary Videos 1–4 and Supplementary Fig. 6), enhancing functionality in a cell line previously considered incompetent.

We evaluated the effects of an initial DMSO treatment on terminal differentiation in two additional contexts. In low-density cultures, HUES8 cells that had differentiated into definitive endoderm failed to differentiate further into Pdx1+ cells, and cell survival was substantially compromised (Fig. 1d,e). An initial 24-h DMSO treatment enhanced pancreatic differentiation in these low-density cultures, yielding 60% Pdx1+ cells, comparable to the efficiency of high-density cultures (Fig. 1d–f). DMSO treatment of low-density HUES8 cultures also promoted further terminal differentiation into C-peptide+ and glucagon+ endocrine cells (Supplementary Fig. 7).

Because Pdx1+ cells may be a source for cell replacement therapy for diabetes18–20, we assessed the effects of DMSO in an induced pluripotent stem cell line derived from a type 1 diabetic subject (DiPS). Although DiPS control cultures efficiently (~60%) differentiated toward definitive endoderm, further differentiation to Pdx1+ cells was inefficient (0.4%) even at high density. An initial 24-h DMSO treatment increased differentiation to Pdx1+ cells by 100-fold, from 0.4% to 44% (Fig. 1d,g).

We tested the effect of DMSO on differentiation across lineages in more than 25 other human pluripotent cell lines, including those used to generate the lineage scorecard2. DMSO treatment enhanced directed differentiation (Fig. 2a) into cells of all three germ layers (Fig. 2b,c).
both as a percentage of total cells (Supplementary Table 1) and in total yield of cells (Supplementary Table 2). The effects of DMSO were particularly large in poorly performing cell lines (Supplementary Fig. 8a). For instance, HUES1 is predicted to have low differentiation potential for ectoderm as a result of suppressed expression of ectodermal-related genes2. DMSO treatment raised the ectodermal differentiation potential of HUES1 from 28% to 93% (Supplementary Fig. 8b,c and Supplementary Table 1). Hence, DMSO treatment raises the minimum differentiation potential across all germ layers and reduces the probability of a very low differentiation rate.

The average cycling times of hESC and hiPSC lines range from ~24 to 48 h (refs. 21,22). Because growth inhibition is associated with cell cycling time, we assessed DMSO effects on differentiation following a 24- or 48-h treatment. We observed that extending treatment from 24 to 48 h improved differentiation for several cell lines and that optimizing the DMSO treatment could result in 80%–90% differentiation in some cases (Supplementary Fig. 9).

In several of the cell lines tested, we observed that DMSO treatment positively affected differentiation further down the endocrine lineage, resulting in enhanced induction of Pdx1+ pancreatic progenitor cells (Supplementary Fig. 10a). Although the effect varied depending on the line, an initial 24-h DMSO treatment enhanced Pdx1+ cell induction on average by 15-fold and 5-fold in hESC and hiPSC lines, respectively (Supplementary Fig. 10b,c). Notably, in cell lines with high differentiation efficiencies to Sox17+ definitive endoderm, DMSO improved differentiation into Pdx1+ cells despite having modest impacts at the initial stages of differentiation. Similarly, in the H1 cell line, DMSO treatment promoted generation of terminally differentiated endocrine cells (Supplementary Fig. 3c,d) to levels comparable to those reported for high-propensity cell lines18,19.

In addition to these persistent effects, preliminary data show that DMSO treatment can improve functionality after transplantation in vivo. Transplantation of control and DMSO-pretreated HUES8 pancreatic progenitor cells under the kidney capsule of SCID-Beige mice showed that an initial 24-h DMSO treatment (prior to directed differentiation in vitro) improved insulin secretion in response to glucose stimulation (data not shown).

We investigated the mechanisms by which DMSO treatment enhances the response of pluripotent stem cells to differentiation signals (Fig. 3). We focused on the HUES6 line for this analysis because it exhibited the greatest improvements in differentiation potential. Consistent with reports in other tissue culture systems13–15, flow cytometry analysis of propidium iodide–stained HUES6 cells showed that a 24-h 2% DMSO treatment almost doubled 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 phase9,23. DMSO treatment was associated with a fourfold increase in immunofluorescence reactivity of hypophosphorylated Rb (Fig. 3b) and substantially reduced levels of the phosphorylated and hyperphosphorylated forms of the Rb protein (Fig. 3c,d). Similarly, for HUES8 cells, higher initial plating density was associated with an increased proportion of cells in G1 (Fig. 3f) and reduced levels of the phosphorylated and hyperphosphorylated forms of the Rb protein (Fig. 3g).

We performed live imaging of HUES6 cells during a 24-h DMSO treatment and subsequent release. DMSO-treated cultures had a slow growth rate and subsequently reached the same degree of confluence as control cultures upon release from DMSO treatment (Supplementary Fig. 11). When cell contact was disrupted, DMSO pretreatment no longer had an impact on differentiation (Fig. 3e), a result analogous to observations in other cell types in which loss of cell-to-cell contact is associated with inactivation of Rb and impaired differentiation24. Prolonged or permanent cell-cycle arrest also inhibited DMSO-enhanced differentiation potential (Supplementary Fig. 12), suggesting that opposing mechanisms may govern spontaneous extraembryonic differentiation25,26 and directed differentiation of pluripotent stem cells.

In summary, the results presented here challenge the view that only some human pluripotent stem cell lines are suitable for differentiation into particular lineages. Culturing hESCs and hiPSCs in DMSO for a brief period (24–48 h) can improve the ability of several cell lines to differentiate into the desired lineage. This method also enhances the prospects of using patient-specific
hiPSCs for disease modeling and autologous cell replacement therapy. Optimizing the duration of the DMSO treatment for a given cell line’s cycling time can further improve differentiation potential (Supplementary Note). Consistent with prior reports\textsuperscript{17}, DMSO concentrations of 1\%–2\% did not have any toxic effects on hESCs and hiPSCs. Moreover, the effects of an initial DMSO treatment persist to improve functionality.

Although 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\textsuperscript{9,10,27–29}. Regulation of Rb activity using DMSO could have applications in many settings.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary information is available in the online version of the paper.

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**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**

The authors declare competing financial interests: details are available in the online version of the paper.
ONLINE METHODS

hESC and 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 MEF-conditioned medium (R&D Systems) with 20 ng/ml bFGF supplemented with 10 µM of the ROCK inhibitor Y-27632 (ref. 30). The 26 cell lines analyzed in the study were: HUES1, 3, 4, 6, 8, 9, 28, 44 and 49; H1, H9 and H14; BG01V/hOG; hiPS 11b, 17b, 18a, 18b, 18c, 27b and 29d; DiPS1 1000 and 1020-9; 1015-3F B; 1013-4FA; iPS Sev A and iPS Sev B.

HUES and hiPSC cell lines were obtained from the Human Embryonic Stem Cell Facility and the iPS Core Facility of the Harvard Stem Cell Institute. The derivation and characterization of the HUES1, hiPS3 and DiPS22 cell lines has previously been reported. Other hiPS cell lines reported in the study were cultured via retroviral or Sendai viral reprogramming with three (Oct4, Sox2 and Klf4) or four (Oct4, Sox2, Klf4 and c-Myc) factors: 1015-3F B (three-factor retroviral), 1013-4FA (four-factor retroviral), iPS Sev A and iPS Sev B (four-factor Sendai viral). The HUES cell lines BG01V/hOG (Gibco), H1, H9 and H14 (WiCell) were also included in the study.

Differenciation protocols. For all differentiation studies, hESC/iPSCs were plated onto wells coated with growth factor–reduced Matrigel (BD Biosciences) in MEF-conditioned medium with 20 ng/ml bFGF and 10 µM of the ROCK inhibitor Y-27632. Plating densities were as follows: 0.25 million cells per well of a six-well plate (low density), 0.5 million per well of a six-well plate (medium density), 1 million per well of a six-well plate (high density), 100,000 per well of a 96-well plate (high density). Multilineage differentiation potential across cell lines was assessed at high density, 24 or 48 h before the onset of differentiation, cells were cultured in MEF-conditioned medium with 20 ng/ml bFGF and 1% or 2% DMSO (with two DMSO treatments for a 48-h treatment time). This medium was removed and replaced with RPMI-B27 (ref. 33). Plating densities were as follows: 0.25 million cells per well of a six-well plate (low density), 0.5 million per well of a six-well plate (medium density), 1 million per well of a six-well plate (high density), 100,000 per well of a 96-well plate (high density). Multilineage differentiation potential across cell lines was assessed at high density, 24 or 48 h before the onset of differentiation, cells were cultured in MEF-conditioned medium with 20 ng/ml bFGF and 1% or 2% DMSO (with two DMSO treatments for a 48-h treatment time). This medium was removed and replaced with one of the following medium at the start of each differentiation, with replacement of medium every day in all protocols.

Ectoderm. Cells were cultured in KO-DMEM (Invitrogen) medium containing 10% knockout serum replacement (Invitrogen) and supplemented with Noggin (500 ng/ml; R&D Systems) and SB431542 (10 µM; Tocris) for 4 d (hESCs) or 2 d (hiPSCs), time points corresponding to the peak expression of Sox1 (ref. 33).

Mesoderm (4-d induction). Cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant activin A (AA; 100 ng/ml; R&D Systems) for 1 d followed by human recombinant Bmp4 (10 ng/ml; R&D Systems) for 3 d.

Mesoderm (1-d induction). As repeated Bmp4 treatment can also promote differentiation into nonmesodermal fates, a second mesodermal induction protocol known to reduce differentiation into alternative fates was also assessed: cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant AA (100 ng/ml) + Wnt3a (20 ng/ml; R&D Systems) for 24 h.

Endoderm. Cells were cultured in MCDB-131 medium (Invitrogen) supplemented with NaHCO3 (2.5 g/l), 1% GlutaMAX, glucose (5.5 mM), 0.1% FAF-BSA (Proliant/Lampire) and ITSX (1:5000; Invitrogen) and treated with Wnt3a (20 ng/ml) + AA (100 ng/ml) for 1 d and then AA (100 ng/ml) for 3 d.

Terminal endocrine differentiation. Cells were cultured in MCDB-131 medium supplemented with NaHCO3 (2.5 g/l), 1% GlutaMAX, glucose (5.5 mM), 0.1% FAF-BSA and ITS: X (1:5000) and were treated with Wnt3a (20 ng/ml) + AA (100 ng/ml) for 1 d, AA (100 ng/ml) for 3 d and FGF7 (50 ng/ml; PeproTech) for 2 d. For the following 13 d, cells were cultured in MCDB-131 medium supplemented with NaHCO3 (2.5 g/l), 1% GlutaMAX, glucose (8 mM), 2% FAF-BSA and ITSX: X (1:200) and treated for 4 d with FGF7 (50 ng/ml) + noggin (100 ng/ml) + retinoic acid (2 µM; Sigma) + SANT-1 (0.25 µM; Sigma) + AA (20 ng/ml); 3 d with SANT-1 (0.25 µM) + PdBu (200 nM; EMD) + noggin (100 ng/ml); and 4–6 d with noggin (100 ng/ml) + Alk5 inhibitor (1 µM; Axxora).

Terminal mesodermal differentiation. Cells were cultured in RPMI-B27 medium (Invitrogen) supplemented with human recombinant AA (100 ng/ml; R&D Systems) for 1 d followed by human recombinant Bmp4 (10 ng/ml; R&D Systems) for 3 d. Medium was then replaced every 2 d for another 8 d with RPMI-B27 without supplementary cytokines. Cells were differentiated into cardiomyocytes following temporal modulation of Wnt signaling.

Terminal ectodermal differentiation. Cells were cultured in KO-DMEM (Invitrogen) medium containing 10% knockout serum replacement (Invitrogen) and supplemented with noggin (500 ng/ml; R&D Systems) + SB431542 (10 µM; Tocris) for 4 d and were allowed to differentiate in noggin alone for the remaining 8 d (ref. 33).

Immunocytochemistry and quantification. Cells were rinsed in PBS and fixed in 4% paraformaldehyde (PFA; Sigma) for 30 min. Following the rinses, cells were blocked for 1 h at room temperature in 5% donkey serum (Jackson ImmunoResearch), 0.3% Triton X-100 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 AF3369), brachyury (mesoderm; R&D Systems AF2085), Sox17 (definitive endoderm; R&D Systems AF1924), Pdx1 (pancreatic progenitor; R&D Systems AF2419), C-peptide (endocrine cell; Cell Signaling 4593), glucagon (endocrine cell; Cell Signaling 2760), nestin (neural; R&D Systems MAB1259), Tuj1 (neural; Covance MMS-435P), troponin C and troponin T (mesodermal and cardiomyocyte; Abcam ab30807 and ab8295), Nkx2.5 (cardiac-specific homeobox protein; R&D Systems MAB2444), Phospho-Rb-Ser780 (Cell Signaling 9307) and hypophosphorylated Rb (1:50 dilution; BD Pharmingen 554164). Cells were rinsed the next day, followed by secondary antibody incubation for 1 h at room temperature at a 1:500 dilution. Secondary antibodies (Invitrogen) conjugated to Alexa Fluor 488 or 594 were used to visualize primary antibodies. Following PBS rinses, all nuclei were visualized by staining with Hoechst 33342 (1:1,000, Molecular Probes).

Quantification. In each of the differentiation experiments, 2–4 wells from the same passage were assessed for differentiation...
potential. With the Cellomics high-content screening system, 30 10× 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 min. Cells were then washed twice in staining buffer (PBS containing 0.2% BSA) (BD Biosciences). For surface-marker staining, 10^5–10^6 cells were resuspended 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, resuspended in 200 µl staining buffer and incubated in 15 µl of 7AAD for live/dead discrimination before 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 and then washed once in cold PBS. Cells were fixed in 250 µl of Cytofix/Cytoperm Buffer (BD Biosciences) for 20 min at 4 °C and washed twice in BD Perm/Wash Buffer Solution (BD Biosciences). Cells were resuspended in 100 µl staining/blocking solution consisting of Perm/Wash buffer with 2% normal goat serum (or serum appropriate for the species of the secondary antibody). Cells were then incubated for 30 min at 4 °C with primary antibodies at empirically predetermined dilutions and washed twice in Perm/Wash buffer. Last, cells were incubated with the appropriate secondary antibodies for 30 min at 4 °C and washed twice before analysis on the BD FACS Canto II. The following primary antibodies were used: goat SOX17 (1:20; R&D Systems), HNF3β (1:20; BD Biosciences), mouse anti-NKX6.1 (1:50; DSHB, University of Iowa); rabbit anti-synaptophysin (1:100; Dako A0010), 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) was added and incubated for 30 min at 4 °C and followed by a final wash in Perm/Wash buffer. Cells were analyzed on the BD FACSCanto II using the BD FACSDiva Software with at least 30,000 events being acquired.

Quantitative RT-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA (100–120 ng) was amplified by PCR using TaqMan Universal Master Mix and TaqMan Gene Expression assays that were preloaded 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 ref. 36.

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

Cell-cycle analysis. Cells were trypsinized and fixed in 100% EtOH for 30 min on ice, treated with RNase A (100 µg/ml, Qiagen) and stained with propidium iodide (PI; 50 µg/ml, Invitrogen) in 0.1% Triton X-100 for at least 30 min. Cells were assessed on the BD Biosciences 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 X-100 for 1 h at room temperature and then incubated with the following primary antibodies overnight at 4 °C: G3-245 (ppRb, 1:1,000, BD Pharmingen), Phospho-Rb-Ser780 (1:200, Cell Signaling), Phospho-Rb-Ser795 (1:200, Cell Signaling), Phospho-RbSer807/811 (1:200, Cell Signaling), 4H1 (total Rb, 1:1,000, Cell Signaling or GAPDH (1:5,000, Abcam) as the loading control. After washing, the membranes were incubated with secondary antibodies for 1 h at room temperature: anti-mouse HRP (1:1,000, Cell Signaling) or anti-rabbit HRP (1:1,000, Cell Signaling), and then incubated in Chemiluminescent HRP substrate (Millipore) for signal detection and development.

Statistical analysis. Means and s.e.m. were determined for the above variables. For statistical comparisons, these values were subjected to unpaired or paired (across multiple cell lines) two-tailed Student’s t-tests. P values ≤0.05 were considered significant.

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