High-throughput fingerprinting of human pluripotent stem cell fate responses and lineage bias

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Populations of cells create local environments that lead to emergent heterogeneity. This is particularly evident with human pluripotent stem cells (hPSCs): microenvironmental heterogeneity limits hPSC cell fate control. We developed a high-throughput platform to screen hPSCs in configurable microenvironments in which we optimized colony size, cell density and other parameters to achieve rapid and robust cell fate responses to exogenous cues. We used this platform to perform single-cell protein expression profiling, revealing that Oct4 and Sox2 costaining discriminates pluripotent, neuroectoderm, primitive streak and extraembryonic cell fates. We applied this Oct4-Sox2 code to analyze dose responses of 27 developmental factors to obtain lineage-specific concentration optima and to quantify cell line–specific endogenous signaling pathway activation and differentiation bias. We demonstrated that short-term responses predict definitive endoderm induction efficiency and can be used to rescue differentiation of cell lines reticent to cardiac induction. This platform will facilitate high-throughput hPSC-based screening and quantification of lineage-induction bias.

hPSCs offer opportunities for drug development, understanding mechanisms of human cell development and cell-based therapies, but these applications require a predictive understanding of factors that control cell fate. Although much progress has been made, there is still a need for improved reproducibility in cell fate–response assays. For example, fibroblast growth factor (FGF)1,2, activin A3,4, leukemia inhibitory factor (LIF)5,6 and Wnt7,8 signaling have all been reported both to maintain pluripotency of hPSCs and to have no effect on pluripotency. The International Stem Cell Initiative recently reported the first multilaboratory comparative study of published defined culture systems for hPSC expansion5; only two out of eight tested media reproducibly maintained hPSCs across laboratories and cell lines, a result highlighting the need to identify and control key confounding factors.

Population context can have dramatic consequences on stem cell maintenance, differentiation and reprogramming. hPSCs exist in complex microenvironments containing multiple factors that regulate cell fate, including endogenous ligands10, extracellular matrix proteins10, mechanical forces11, cell-cell contact11 and cell subpopulations2,10. Critically, these factors are spatially heterogeneous10, and such heterogeneity introduces substantial variances in cell response. Indeed, reprogramming to pluripotency12, differentiation toward neural13, pancreatic14 and cardiac15 cell types, and the disease phenotype of a familial dilated cardiomyopathy–induced hPSC model16 all have specific organizational and density-dependent optima.

We have developed a suite of tools to test hPSC responses to exogenous factors and to categorize cell lines on the basis of differentiation bias. The platform consists of a method to pattern cells in 96-well plates, an optimized cell-fate factor screening assay protocol, defined media and substrate, and a single-cell imaging and data analysis pipeline. We optimized colony size, cell density, media composition and substrate to allow robust cell fate responses to exogenous cues to be measured at 48 h. Characterization of single-cell protein expression across diverse induction conditions revealed that Oct4 and Sox2 costaining could discriminate pluripotent, neuroectoderm, primitive-streak and extraembryonic subpopulations, enabling fingerprinting of cell-line response to lineage-induction stimuli. These responses were stable between passages, variable between cell lines and predictive of lineage-induction efficiency, and they could be used to improve differentiation of reticent cell lines. Additionally, we applied this platform to characterize 27 developmental signaling factors selected by the International Stem Cell Initiative across a wide range of doses, revealing new dose- and lineage-specific optima.

RESULTS
Microenvironmental control for hPSC screening

We previously developed an assay in which hPSC distribution (colony size, shape and spacing) can be controlled by microcontact printing (µCP) of substrates onto slides. This work indicated that colony size control could be an important parameter in...
To adapt this technique for high-throughput studies, we developed a method of μCP in which substrates are directly printed into 96-well plates in user-defined patterns (Supplementary Fig. 1) in defined serum-free medium (SF) and substrate (a fibronectin-and-gelatin mixture, FnGel) (Supplementary Fig. 2). We dispense single-cell suspensions into the wells, allow the cells to settle and adhere to the patterned substrate for 6 h, wash away nonadherent cells, add test factors to the wells for 42 h and then fix and analyze the plates (Fig. 1a). Each well contains an array of hPSC colonies, and we use automated microscopy to obtain single-cell data such as x and y coordinates and protein expression levels (Fig. 1b).

On the basis of our current understanding of the relation between colony size and endogenous signaling\textsuperscript{10}, we reasoned that calibration of colony size would allow robust detection of both positive and negative regulators of pluripotency. To test this, we assayed the response of hPSC (H9 line) colonies of different sizes to five control conditions: murine embryonic fibroblast-conditioned medium (CM), base medium alone (SF), SF with FGF2 and SB431542 (FTi), SF with BMP4 (B), and SF with BMP4 and activin A (BA). *P < 0.01, ANOVA with Tukey post hoc test. Error bars, s.d. (n = 3).

Oct4 and Sox2 expression discriminate early cell fates

We next sought to determine whether early lineage specification could be quantified using the 96μCP platform. We chose six previously characterized conditions: SF; CM (maintains pluripotency\textsuperscript{17}); SF with heregulin-β1, activin A and FGF2 (HAF; maintains pluripotency\textsuperscript{18}); B (induces trophectoderm and primitive endoderm\textsuperscript{19–21}); BA (induces primitive streak\textsuperscript{22}); and FTi (induces neuroectoderm\textsuperscript{22,23}). For all conditions, we assessed single-cell protein expression of a panel of early development lineage markers including pluripotency (Oct4, Sox2, Nanog, Tra-1-60), primitive streak (brachyury, Gata4, Snail), neural (Pax6, Sox1, Sox3), extraembryonic (Sox7, Cdx2, Hand1), endo
derm (Cxcr4, Foxa2) and mesoderm markers (brachyury, Gata4) (select images in Fig. 2a). Several later-stage markers, typically arising more than 6 d post-induction, including Pax6, Sox1, Sox3, Sox7, Cdx2, Hand1 and Cxcr4, showed no difference in expression, indicating that at this early test point (42 h), these markers are not differentially expressed, as expected.

Two-dimensional hierarchical clustering of marker expression levels across the six control conditions (Fig. 2b) confirmed that different induction conditions result in distinct protein expression profiles. Thresholds for positive expression were determined for each protein on the basis of differential expression across conditions (Fig. 2c). SF and FTi conditions clustered together, indicating that under basal conditions the hPSCs are largely neuroectoderm fated, a finding in line with previous observations\textsuperscript{24}. Oct4−Sox2\textsuperscript{−}, previously shown to mark committed neural precursors in human\textsuperscript{25} and mouse\textsuperscript{26} development, was seen exclusively in the neuroectoderm-inducing conditions FTi and SF. Oct4−Sox2\textsuperscript{−} cells clustered separately from the other groups, which we interpreted as marking a nonpluripotent, non-neural, non–primitive streak population likely fated toward extraembryonic tissue (trophectoderm and primitive endoderm). Primitive-streak markers also clustered together, and, intriguingly, Oct4\textsuperscript{+}Sox2\textsuperscript{−} expression clustered with this group. We observed that, across all control conditions, Oct4\textsuperscript{+}Sox2\textsuperscript{−} was exclusively found in the BA condition, which induces primitive streak (Fig. 2d,e) and is associated exclusively with high expression levels of Snail, brachyury and Gata4 (Fig. 2f).

These data support the use of Oct4 and Sox2 as a binary code to discriminate four major early cell fates in human development: Oct4\textsuperscript{+}Sox2\textsuperscript{−} for pluripotency, Oct4\textsuperscript{−}Sox2\textsuperscript{−} for early neuro
cectoderm, Oct4\textsuperscript{+}Sox2\textsuperscript{−} for early primitive streak and Oct4\textsuperscript{−}Sox2\textsuperscript{−}
for early extraembryonic committed and other tissues (Fig. 2g).
This classification is congruent with previous reports of Oct4 and Sox2 expression in these lineages19,27–29.

Analysis of early hPSC cell fate responses

We used the 96µCP platform and the Oct4–Sox2 code to simultaneously characterize early cell fates in response to 27 developmental factors. Responding factors (Fig. 3a; nonresponding factors in Supplementary Fig. 6) were classified as promoting pluripotency, neuroectoderm, primitive streak or extraembryonic/other or as having a bimodal effect (inducing different subpopulations at different concentrations). We have summarized the classification of these factors as well as a recommended concentration for use in chemically defined media in Table 1.

FGF2 (ref. 3), TGF-β1 (ref. 30), activin A30, heregulin-β1 (ref. 18), IGF1 (ref. 18) and noggin31 all promoted pluripotency, as predicted from the literature. In contrast, a previously published 7-d nonpatterned hPSC-based screen of 806 human extracellular factors assaying for pluripotency regulators found that only FGF2 and pigment epithelium–derived factor maintained pluripotency—with TGF-β1, activin A, heregulin-β1, IGF1, noggin and all other factors tested resulting in streak, in contrast to activin A alone, which yielded no primitive streak34. LDN-193189 (LDN; ref. 35), an inhibitor of BMP receptor ALK2/3/6 previously uncharacterized in hPSCs, increased neuroectoderm. To determine an optimal dose to inhibit BMP signaling, we obtained the LDN dose curve in the presence of BMP4 (Fig. 3b). LDN increased neuroectoderm both with and without BMP4 in a dose-dependent manner up to 10 µM LDN. Interestingly, LDN rescued the pluripotency-suppressing effect of BMP at LDN concentrations up to 2.5 µM; however, at higher concentrations pluripotency decreased. Nostro et al. have shown that during endoderm induction from hPSCs, there is a cell line–specific need to inhibit BMP signaling36. We therefore added 2.5 µM LDN during stage 2 of the Nostro et al. protocol and confirmed a significant induction of later-stage endodermal PDX1+ pancreatic progenitor cells (P = 0.0006; Fig. 3c). These results indicate that findings from our high-throughput assay are congruent with results from traditional assays and can yield predictive signaling insights about later stages of differentiation.

It has previously been reported that DKK1 has little or no effect on hPSC pluripotency3. However, these studies were performed on hPSCs cultured on fibroblast feeders, measuring formation of alkaline phosphatase–expressing colonies after
26 d. In our assay, DKK1 had a surprising effect of inhibiting pluripotency and enhancing neuroectoderm. We speculate that endogenous supporting factors produced by the feeders mask this effect of DKK1. Indeed, when we tested the response of endogenous supporting factors produced by the feeders during stage 2 of endoderm differentiation, hPSCs (H9 line) were stained with DAPI and for PDX1. Scale bar, 100 μm. The percentage of PDX1+ cells is quantified in the plot (right). *P = 0.0006 (ANOVA). Error bar, s.d. (n = 3).

Quantification of cell-line differentiation propensities
It has long been understood that hPSCs have varying differentiation propensities. We tested whether the 96µCP platform would allow a rapid evaluation of cell line–specific differentiation tendencies by measuring fate responses to six conditions (SF, CM, B, BA, FTi, and SF with PD0325901) for 21 cell samples. Our test panel consisted of 15 human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) lines, including two in-house–generated hiPSC lines, ZAN3i-85UCBT (ZAN3) and ZAN11i-85UCBT (ZAN11) (Supplementary Fig. 7 and Online Methods); an in-house–generated karyotypically abnormal H7 cells (H7*) with trisomy 12, which is recurrent in hPSC cultures; differentiated H9 cells (H9diff); and multiple passages of H9 and ZAN11. We performed two-dimensional hierarchical clustering on this data set (6 controls × 4 subpopulation measurements = 24 data points for each cell line), which enabled visualization of cell-line similarities (Fig. 4a). The H7* line expressed all pluripotency markers and had normal hPSC morphology but was the only line that failed to differentiate in response to BMP4, which is indicative of an acquired abnormality (Fig. 4b and Supplementary Fig. 8). The three passages of H9 and ZAN11 cells clustered closely; such clustering indicates that the response profile is stable over multiple passages and that the assay is reproducible.

We sought to determine whether our 48-factor response profiles could be used to improve differentiation protocols for specific cell lines. Despite the overall similar differentiation profile...
of ZAN3 and ZAN11 lines (Fig. 4a) and similar primitive-streak induction frequency in response to BMP and activin A, when activin A was removed, ZAN3 cells increased primitive-streak induction, in contrast to ZAN11 and H9 cells, in which primitive-streak induction decreased. This trend was confirmed over multiple passages (ANOVA, \( P = 0.005 \)) (Fig. 4c). To further support the primitive-streak commitment of these lineages and to associate this early cell fate with functional commitment, we compared the cardiac differentiation efficiency of ZAN11 and ZAN3 in an 18-d protocol38. We varied activin A concentration during the first 4 d, as differentiation is known to be sensitive to activin A in a cell line–dependent manner during this period39. As predicted by the day 2 results (Fig. 4c), day 18 cardiac troponin T (TnT) expression varied with activin A (\( P = 0.005 \), two-factor ANOVA with \( n = 2 \), independent passages), with additional activin A increasing cardiomyocyte (TnT\(^{+}\)) output in ZAN11 but decreasing cardiomyocyte output in ZAN3 (both >95\% confidence using linear regression; Fig. 4c).

To further examine how predictive the day 2 96\(\mu\)CP response profiles were of differentiation over longer periods, we differentiated a panel of 12 hESC and hiPSC lines toward Foxa2\(^{-}\)Sox17\(^{+}\) definitive endoderm using a 5-d induction protocol (Fig. 4d and Supplementary Fig. 9a). Using the ratio of percent primitive streak induced in the BA control to percent extraembryonic in the CM condition (‘mesendoderm prediction index’), we found a significant correlation between this day 2 prediction index and cell-line definitive endoderm induction efficiency at day 5 (correlation coefficient \( r = 0.89, P = 0.0001; \) Fig. 4d and Supplementary Fig. 9a,b). In contrast, pluripotency of the input cell population was not correlated with definitive endoderm induction (\( r = 0.29, P = 0.42; \) Supplementary Fig. 9c).

Quantification of cell-line endogenous signaling

Our results thus far and several recent studies indicate that variability in endogenous signaling is one major source of cell-line differentiation variability. Cell-line- and passage–specific changes in endogenous activin A39 and Wnt24 signaling are known to dramatically reduce cardiac induction efficiency. We reasoned that, by measuring responses to saturating agonists and antagonists of specific pathways, endogenous signaling levels could be assessed and quantitatively placed along a spectrum of low activation (endogenous response equivalent to pathway inhibitors) to high activation (endogenous response equivalent to saturating agonists).

We determined from dose curves the saturating concentrations of factors that affect early cell fate decisions (Fig. 3a). Using H9 cells, we then tested the response to agonists and antagonists of the major pathways regulating hPSC fate (Fig. 4e). To obtain an estimate of activation of an endogenous pathway relative to its dynamic range (percent dynamic range), we combined agonist, antagonist and baseline control measurements (Online Methods equation (1)). The resulting endogenous signaling profile for activin, FGF, EGF, Wnt and BMP activation in H9 is shown in Figure 4e. We used similar quantitative profiles to compare H9 to ZAN11 and ZAN3 lines, revealing that EGF, FGF and activin are all differentially endogenously activated (Fig. 4f and Supplementary Fig. 10). Comparisons of control and pathway agonist-antagonist response profiles over multiple passages indicate high correlation between passages (ZAN11 \( r = 0.90 \) and ZAN3 \( r = 0.88 \)) and lower correlation between cell lines (ZAN11 vs. ZAN3 \( r = 0.52 \)) (Fig. 4g). In summary, cell lines differ in their response to induction conditions and specific pathway agonists and antagonists, and this response is stable between passages. These response-profile measurements offer a quantitative tool for rapidly fingerprinting hPSC signaling profiles.

DISCUSSION

Heterogeneity in cell response in clonal populations arises from many cell-autonomous and non–cell-autonomous factors. Our previous work10 and other diverse evidence led us to hypothesize that key sources of microenvironment variance and optimizing colony size and density would allow robust cell response and thus overcome existing limitations in hPSC high-throughput assays. In comparison to previous assays, our 96\(\mu\)CP platform eliminates the need to seed in CM, results in less hPSC cell fate response variance and faster response kinetics and, in
Figure 4 | Quantitative assessment of cell line–specific endogenous signaling and differentiation. (a) Hierarchical clustering of cell-line responses to six conditions: SF, CM, B, BA, FTI, and SF with P00235901 (Euclidean distance similarity metric, average linkage clustering). 0, Ocs4; S, Sox2; H7, H7 cells with trisomy 12; H9diff, differentiated H9 cells. “p” indicates the passage number for cells lines H9 and ZAN11. Left, sample similarity tree with samples clustered using a distance threshold of 1.4. Clusters are numbered in red. (b) Percentages of four indicated subpopulations, determined via the Oct4-Sox2 code, in response to the indicated stimulus conditions (abbreviations as in Fig. 1). Error bars, s.d. (n = 3). (c) Top, percent primitive streak (day 2) under the indicated conditions in multiple cell lines. Effect of activin A varies across cell lines, P = 0.005 (ANOVA). Error bars, s.d. (n = 3). Bottom, troponin T (TnT) expression (day 18) with varying activin A concentration. Activin A has differential effects on cardiac TnT expression in ZAN3 and ZAN11 cell lines (P = 0.005, two-factor ANOVA with n = 2, independent passages), with activin A increasing primitive streak in ZAN11s and decreasing primitive streak in ZAN3s (both >95% confidence using linear regression). (d) Top, definitive endoderm induction efficiencies of 12 cell lines. Bottom, correlation of cell-line mesendoderm prediction index values from day 2 to actual definitive endoderm induction efficiencies at day 5 for a panel of cell lines (r = 0.089, P = 0.0001). (e) Left, response of the H9 line to signaling pathway agonists and antagonists. Error bars, s.d. (n = 3). Right, estimated endogenous signaling levels of specific pathways for H9 (Online Methods equation (1)). (f) Estimated endogenous signaling levels for ZAN3 and ZAN11 cell lines. (g) Passage-to-passage correlation of endogenous signaling profiles. The 60 response outputs (4 readouts × 15 conditions) were obtained for multiple passages for ZAN11 and ZAN3.

conjunction with Oct4 and Sox2 marker analysis, allowed us to screen for cell fate responses to 27 developmental factors, thereby providing the most comprehensive characterization of hPSC cell fate decisions to date.

Our 48-h high-throughput stem cell response-signature approach is a direct measurement of early transcription factor expression changes in response to diverse exogenous cues. In combination with cell line–specific epigenetic and gene expression data40, this may offer a strategy to decipher the genetic and epigenetic basis of cell line–specific responses to cues. Additionally, hPSC lines can now be generated at a higher rate than they can be characterized. We propose the 96UCLP platform as an effective first-pass assay to detect tumorigenic cell lines and quantify neuroectoderm and primitive-streak differentiation propensity. Further development may lead to a simple and effective in vitro teratoma surrogate assay.

Finally, population heterogeneity arising from microenvironmental differences applies broadly to adherent cell populations and is increasingly recognized as a major obfuscating factor in drug-screening campaigns41. We believe that the methods presented herein are widely applicable to adherent cell types. We have developed 96UCLP based assays for mouse epiblast stem cells and hPSC-derived cardiac and endoderm cells with minimal modification to the base medium, substrate and pattern size. Applying these concepts toward rapid characterization of the signaling involved in cell fate decisions of differentiated cell types is a promising strategy to accelerate the drive toward clinical regenerative medicine and drug screening of hPSC disease models.

METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
E.J.P.N. designed, performed and analyzed most experiments. J.E.E.O. assisted with immunochemistry and software development. P.B.L. performed cardiac induction experiments. S.S. created hiPSC lines. T.Y. performed endoderm induction experiments. M.M.A. and T.Y. provided cell culture support. S.K.W. provided editorial input on the manuscript. E.J.P.N. and P.W.Z. designed the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture. hESC lines H9, H1 and H7 were obtained from the WiCell Research Institute. ZAN3 and ZAN11 were derived from activated CD3+ T cells enriched from umbilical cord blood (see below). HES2 (hESC) was provided by G. Keller (McEwen Centre for Regenerative Medicine/University Health Network). BJ1D (hiPSC) was provided by M. Radics (University of Toronto). 110 (hiPSC) and CA1 (hESC) were provided by A. Nagy (Samuel Lunenfeld Research Institute). PDX1 (MEL1-derived PDX1-GFP hESC) was provided by D. Melton (Harvard University). Runx1 (HES3-derived Runx1-GFP hESC) was provided by A. Elefanty (Monash University). R306C44, RTT-Δ3-4 #37 and T158M #5 (ref. 45), all Rett syndrome hiPSC disease models, as well as BJ4YA (hiPSC) were provided by J. Ellis (The Hospital for Sick Children). H9, H1, H7, ZAN3, ZAN11 and PDX1 cells were routinely cultured on feeder layers of irradiated MEFs feeders in knockout (KO) Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) with 20% KO-serum replacement (Invitrogen) (KO-DMEM) and supplemented with 4 ng ml−1 FGF-2 (PeproTech). Cells were passaged 1:4–1:6 every 4–5 d and were dissociated into small clumps using 0.1% collagenase IV (Invitrogen). HES2 and Runx1 were cultured on growth factor reduced Matrigel (BD Biosciences) in DDH2O for 1 h.

Microcontact printing of substrate into 96-well plates. PDMS stamps were fabricated using standard soft lithography techniques46, with the exception that liquid PDMS was cast into a Teflon mold before curing so as to allow control of the shape of the PDMS stamp (Supplementary Fig. 1). PDMS stamps were cast, consisting of rectangular base with 24 posts (6 × 4) with micropatterned surfaces configured to enable microcontact printing directly into 96-well plates. The microcontact printing follows a protocol employed previously46. Substrate solution consisted of either MG diluted 1:30 in phosphate-buffered saline (PBS) or a solution of 0.00125% fibronectin (Sigma-Aldrich, F1141) and 0.002% gelatin (Sigma-Aldrich, G9391). Substrate solution was deposited onto the patterned surface of ethanol-sterilized PDMS stamps for 4 h at room temperature. Stamps were rinsed with ddH2O, dried gently with N2 gas and placed into tissue-culture treated 96-well plates (Costar). Stamps were incubated in the 96-well plates for 7–10 min in a humidity chamber (relative humidity 55–70%). The stamps were then removed, and substrates were passivated with 5% weight Pluronic F-127 (Sigma-Aldrich) in ddH2O for 1 h.

Seeding hiPSCs onto patterned substrates. hiPSCs were dissociated using TrypLE for 3 min. TrypLE was inactivated by adding medium containing 20% KO-serum replacement (SR) (Invitrogen). Cells were centrifuged and resuspended in either XVIVO10 (XV) or SF media, both supplemented with 40 ng ml−1 bFGF (R&D), 10 ng ml−1 activin A (R&D), 10 µM ROCK inhibitor Y-27632 (Tocris) and 7 µg ml−1 additional insulin. SF medium is a modification of DC-HAIF18 and consists of DMEM/F12, 1× non-essential amino acids, 50 U ml−1 penicillin, 50 µg ml−1 streptomycin, 10 µg ml−1 bovine transferrin, 0.1 mM β-mercaptoethanol (all Invitrogen), 2% fatty acid–free Cohn’s fraction V BSA (Serologicals), 1× Trace Elements A, B and C (Mediatech), 50 µg ml−1 ascorbic acid (Sigma) and 7 µg ml−1 recombinant human insulin. XV includes XVIVO10 (Lonza) supplemented with 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1× non-essential amino acids. Cells were seeded at 105 cells per well (or as described in text) and incubated. After 6 h, cells were washed with PBS two times and incubated a further 42 h with fresh medium as indicated (SF supplemented with factors, or MEF-CM).

Immunocytochemistry and image analysis. Plates were fixed for 30 min in 3.7% formaldehyde and permeabilized for 3 min in 100% methanol. Plates were imaged and quantitatively analyzed using the Cellomics Arrayscan VTI platform and Target Activation algorithm (Thermo Scientific). This algorithm generates nuclear masks and provides single-cell nuclear intensity values for protein expression (Oct4, Sox2, etc.), DNA content through DAPI (4′,6-diamidino-2-phenylindole) staining, and spatial x and y coordinates of the nuclei centroids. Insufficiently patterned wells were excluded from analysis on the basis of a predetermined number of cells per well. To aid in pattern visualization and quality control, we developed a publicly available Matlab script to plot these x and y coordinates for all wells in a 96-well plate (E. Nazareth, Analysis and visualization of Cellomics data, Matlab Central File Exchange: http://www.mathworks.com/matlabcentral/fileexchange/43107/). Clustering of cells into colonies using Euclidean distance was performed using clusterData (B. Shoelson, clusterData, Matlab Central File Exchange: http://www.mathworks.com/matlabcentral/fileexchange/35014-clusterdata/).

Fluorescence images were obtained of Oct4 (1:500, BD), Sox2 (1:500, R&D Systems), Nanog (1:500, Cell Signaling), Tra-1-60 (1:500, R&D Systems), brachyury (1:200, R&D Systems), Snail (1:200, R&D Systems) and GATA4 (1:200, R&D Systems). Primary antibodies were incubated overnight in 10% FBS in PBS at 4 °C. Alexa Fluor secondary antibodies (1:500, Molecular Probes) were incubated for 1 h in 10% FBS in PBS at room temperature. 16-bit TIFF images were obtained for each channel, contrast adjustment was performed identically across all controls and channels were combined into pseudocolored composite images.

hiPSC derivation. Umbilical cord blood samples were collected from consenting donors according to ethically approved procedures at Mt. Sinai Hospital (Toronto). Activated CD3+ T cells enriched from umbilical cord blood were reprogrammed as described previously47. T cells were enriched from umbilical cord blood using an EasySep human T cell–enrichment kit (Stemcell Technologies; cat# 19051). They were expanded for 4 d using Dynabeads human T-activator CD3/CD28 beads (Invitrogen; cat# 111-61D) and 30 U per ml recombinant human IL-2 (R&D; cat# 202-IL-010) in OpTmizer T cell–expansion serum-free medium (Invitrogen; cat# A1048501). Mutant Sendai virus delivering human OCT4, SOX2, KLF4 and MYC (kindly provided by DNAVEC Corporation) was added to the cells at MOI 20 on day 5. T-cell medium was replenished on day 6, and the cells were plated onto irradiated MEF feeders on day 7 in hPSC medium with...
5 ng ml\(^{-1}\) bFGF. Medium was exchanged every 48 h until hiPSC colonies were picked and characterized 3 weeks later.

**hiPSC cardiomyogenic and endoderm induction.** hiPSC cardiomyogenic induction was performed using a serum-free, aggregate-based strategy described elsewhere\(^48\). A single-cell suspension of hiPSCs was centrifuged into 400-µm-sized AggreWell inserts (Stemcell Technologies) at a density of 500 cells per microwell, and cells were allowed to aggregate overnight. On day 1, mesoderm formation was induced using 5 ng ml\(^{-1}\) bFGF and varying concentrations of activin A as indicated in Figure 4c. On day 4, cells were transferred to LowCluster plates (Nunc), and further differentiation toward the cardiac lineage was induced with 10 ng ml\(^{-1}\) VEGF and 150 ng ml\(^{-1}\) DKK1 for 4 d. Subsequently, cells were maintained in 10 ng ml\(^{-1}\) VEGF and 5 ng ml\(^{-1}\) bFGF. Custom defined medium provided in kind by G. Keller was used as the base medium. Cells were kept under hypoxic conditions (5% O\(_2\)) from days 0–12 and then transferred to normoxic conditions. Endoderm induction into PDX1\(^+\) pancreatic progenitor cells was performed as described by Nostro et al.\(^{36}\). Endoderm induction into Foxa2\(^+\)Sox17\(^+\) definitive endoderm was performed as described by Rezania et al.\(^{49}\) in 96-well plates.

**Flow cytometry.** hiPSC-derived cardiomyocyte aggregates were incubated in collagenase type II (1 mg ml\(^{-1}\); Worthington, LS004176) in Hanks’ Balanced Salt Solution overnight at room temperature and pipetted vigorously to obtain a single-cell suspension, which was then fixed in 4% paraformaldehyde overnight at 4 °C. Cells were permeabilized using IntraPrep Permeabilization Reagent (Immunotech, A07803). Cardiac troponin T primary antibody (Thermo Scientific, MS-295-P) was used at 1:200, and Alexa Fluor 647 donkey anti-mouse IgG secondary antibody (Molecular Probes, A31571) at 1:200. Cells were analyzed using a FACSCanto (BD Biosciences) flow cytometer.

**Estimation of endogenous pathway activation.** To estimate endogenous pathway activation, we obtained the cell fate response to the pathway agonist and antagonist, both at saturating levels as determined by dose curves. The baseline response (in SF basal medium alone) could then be calibrated within this range using the following equation:

\[
\% \text{dynamic range} = \frac{X_{\text{SF Ctrl}} - X_{\text{Antagonist}}}{X_{\text{Agonist}} - X_{\text{Antagonist}}}
\]

(1)

**Statistical analysis.** Statistics were computed using one-way analysis of variance (ANOVA), two-factor ANOVA or linear regression as indicated. Error bars on plots represent s.d. of three or more replicate wells except where indicated differently. All statistics were computed in Matlab using \(P\) values as indicated. Hierarchical clustering was performed with MeV (MultiExperiment Viewer, http://www.tm4.org/mev.html) using Euclidean distance as the similarity metric (centered) and centroid linkage as the clustering method.

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