A Multi-Lineage Screen Reveals mTORC1 Inhibition Enhances Human Pluripotent Stem Cell Mesendoderm and Blood Progenitor Production

Emanuel Joseph Paul Nazareth, Nafees Rahman, Ting Yin, and Peter William Zandstra

1Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON M5S 3G9, Canada
2Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada
3Medicine by Design, University of Toronto, Toronto, ON M5S 3G9, Canada

*Correspondence: peter.zandstra@utoronto.ca
http://dx.doi.org/10.1016/j.stemcr.2016.04.003

SUMMARY

Human pluripotent stem cells (hPSCs) exist in heterogeneous micro-environments with multiple subpopulations, convoluting fate-regulation analysis. We patterned hPSCs into engineered micro-environments and screened responses to 400 small-molecule kinase inhibitors, measuring yield and purity outputs of undifferentiated, neuroectoderm, mesendoderm, and extra-embryonic populations. Enrichment analysis revealed mammalian target of rapamycin (mTOR) inhibition as a strong inducer of mesendoderm. Dose responses of mTOR inhibitors such as rapamycin synergized with Bone Morphogenetic protein 4 (BMP4) and activin A to enhance the yield and purity of BRACHYURY-expressing cells. Mechanistically, small interfering RNA knockdown of RAPTOR, a component of mTOR complex 1, phenocopied the mesendoderm-enhancing effects of rapamycin. Functional analysis during mesoderm and endoderm differentiation revealed that mTOR inhibition increased the output of hemogenic endothelial cells 3-fold, with a concomitant enhancement of blood colony-forming cells. These data demonstrate the power of our multi-lineage screening approach and identify mTOR signaling as a node in hPSC differentiation to mesendoderm and its derivatives.

INTRODUCTION

Human pluripotent stem cells (hPSCs) and their differentiated derivatives offer the exciting opportunity to develop tools to study and treat human diseases. However, robust and reproducible control of hPSC fate remains challenging. Small molecules offer one approach to control hPSC fate, and the discovery and characterization of these compounds can be facilitated by cell-based phenotypic high-throughput screening (HTS). Emerging data from hPSC assays has revealed variable and contradictory observations, even with matched cell lines and protocols (Haibe-Kains et al., 2013). Although the factors underlying this variability are not completely known, population context has been identified as a main contributor to assay inconsistency (Snijder et al., 2012). Spatially heterogeneous (Peerani et al., 2007) micro-environmental factors such as endogenous ligands, extra-cellular matrix proteins (ECMPs), and cell subpopulations are strong regulators of hPSC fate. Specifically, spatial cell distribution has been shown to affect hPSC self-renewal (Maherali and Hochedlinger, 2008), differentiation trajectories in both normal and patient-derived cells (Cai et al., 2009; Chambers et al., 2009), and disease phenotypes (Sun et al., 2012).

Consequently, robust assays that combine defined culture conditions with comprehensive analysis of cell responses to exogenous cues are needed. To this end, we developed a chemically defined cell patterning-based high-throughput (HTP) assay, engineering colony size, local cell density, medium composition, and substrate for rapid and robust measurement of hPSC fate responses to exogenous cues (Nazareth et al., 2013). We applied the assay to screen a library of kinase inhibitors for effects on four early hPSC fates. For each compound, the change in yield and purity in the resulting pluripotent, neuroectoderm (NE), mesendoderm, and extra-embryonic populations were simultaneously tracked, allowing for estimation of selection and induction events. Our analysis identified mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin, as having a strong mesendoderm-inducing effect on hPSCs. Rapamycin was subsequently shown to synergize with bone morphogenetic protein 4 (BMP4) and activin A to enhance BRACHYURY induction more than 3-fold, an effect that propagated to equivalent enhancements of hemogenic endothelium and blood progenitor cells. This study demonstrates the advantages of controlling micro-environmental parameters and measuring multiple subpopulation outputs in parallel on PSC fate screening assays. This strategy should enhance discovery in more complex and predictive multi-cell population drug-screening assays.

RESULTS

A Kinase Inhibitor Screen of hPSCs Revealed Lineage-Specific Regulators

We previously developed a 48-hr hPSC screen that employs control of spatial cell patterning to configure the hPSC micro-environment for rapid and robust response

...
to exogenous cues (96µCP assay) (Nazareth et al., 2013) (Figure 1A). Single-cell OCT4 and SOX2 containing enables simultaneous classification of pluripotent (OCT4+SOX2+), NE (OCT4-SOX2+), mesendoderm (OCT4-SOX2+), and extra-embryonic/other (OCT4-SOX2+) cell fates. This platform can be used to screen test factor effects on yield (percentage) and purity (absolute number of cells) of each subpopulation per colony using in-house software (Figure 1B).

We applied this platform to screen a collection of 400 small-molecule kinase inhibitors at two concentrations (0.2 µM and 1 µM). To ensure a significantly large sample size, we performed further analysis only on compounds which yielded >800 imaged cells per well, resulting in 707 unique conditions including five controls on each plate: base medium (blank), PSC supporting mouse embryonic fibroblast (MEF)-conditioned (CM), NE inducing transforming growth factor β (TGF-β) inhibitor and fibroblast growth factor 2 (FGF2) (TiF), mesendoderm inducing BMP4 with activin A (BA), and extra-embryonic inducing BMP4. The overall distributions of the compounds with respect to each subpopulation indicated that mesendoderm and extra-embryonic/other inducing compounds were underrepresented (Figure 1C). Control conditions showed high reproducibility across plates (Figure S1A). Calculation of the Z′ factor, a statistical parameter used to compare HTP assays (Zhang et al., 1999), for the pattern-based (Z′ = 0.94) and non-pattern-based (Desbordes et al., 2008) (Z′ = 0.29, p < 0.01) assays supported the rationale for controlling micro-environments to improve robustness (Figures S1B and S1C). Sample images of control conditions and selected responders stained for DNA (DAPI, blue), OCT4 (green), and SOX2 (red). BMP4 is the OCT4+SOX2+ control, FGF2 + SB431542 is the OCT4-SOX2+ control, and BMP4 + activin A is the OCT4-SOX2− control. Colony outlined with dotted white line. Scale bar, 100 µm.

Figure 1. HTS of Small-Molecule Regulators of hPSC Pluripotency, Primitive Streak, NE, and Extra-embryonic/Other Cell-Fate Decisions

(A) Assay design. hPSCs are micro-patterned on micro-contact printed 200-µm diameter extracellular matrix proteins (ECMP) circles in 96-well plates. Control or base medium with test compounds is added to each well. After 48 hr, imaging is performed and single-cell OCT4 and SOX2 expression levels are quantified.

(B) Subpopulation purity and high yield are measured to determine inductive effects of compounds. Diagram demonstrates the changes in composition and purity of the "pink" cell population.

(C) Compound distribution ranked by induction efficiency of pluripotency, NE, primitive streak, and extra-embryonic/other cell fates.

(D) Sample composite colony images from controls and selected responders stained for DNA (DAPI, blue), OCT4 (green), and SOX2 (red). BMP4 is the OCT4+SOX2+ control, CM is the OCT4-SOX2+ control, FGF2 + SB431542 is the OCT4-SOX2+ control, and BMP4 + activin A is the OCT4-SOX2− control. Colony outlined with dotted white line. Scale bar, 100 µm.

(E) Calcein (viability) staining of unfixed cells at 48 hr treated with hit compounds. Non-viable control treated with methanol. Colonies are outlined with a dotted white line. Scale bar, 200 µm.

(F) 67 putative hit compounds ranked by increasing viability. n = 3 independent biological replicates. Error bars indicate SD. See also Figure S1.
number of cells of each subpopulation per colony per well. For example, to visualize effects on the PSC population we subtracted the percentage and number of OCT4+SOX2+ cells obtained in the blank to obtain ΔOCT4+SOX2+ (%) and ΔOCT4+SOX2+ (number of cells per colony), respectively (Figure 2A). Based on the subpopulation positive controls, we set thresholds for yield and purity to obtain a list of “responders.” Similar visualizations are shown for NE (OCT4+SOX2+) (Figure 2B) and mesendoderm (OCT4+SOX2−) (Figure 2C). Enlarged plots of the threshold regions showing compound names of responders are shown in Figure S2. Visual inspection of responding compounds confirmed the expected yield and purity effects (Figure 1D). Based on these results, we sought to determine whether responding compounds were enriched for specific inhibitor targets.

**Specific Kinase Inhibitor Targets for NE and Mesendoderm Induction Identified**

To identify fate-modulating pathways enriched in our library, we performed a hypergeometric test to obtain p values for the enrichment of each kinase target in the PSC, NE, and mesendoderm responder groups (five most enriched targets are shown in Figures 2D–2F; count of unique compounds for each kinase target is shown in Figure S3A; count of kinase targets in responders and total screen is shown in Table S1). For the PSC-enhanced cluster no target family was statistically enriched, indicating that the tested kinase inhibitors do not robustly rescue hPSCs from differentiation in the blank lacking exogenous FGF2 and activin A (Vallier et al., 2005). This suggests that, in contrast to differentiation, exogenous pathway agonists, or a combination of inhibitors, may be required to maintain pluripotency in vitro (Tsutsui et al., 2011). For NE responders we obtained the highest enrichment for small molecules targeting the ERK/MEK pathway (p = 2.9 × 10−5) (Figure 2E), as well as a high enrichment (p = 0.001) of small molecules targeting TGF-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Inhibition of TGF-β signaling has been well established to result in hPSC differentiation to NE (Smith et al., 2008; Vallier et al., 2004). We previously observed low levels of endogenous TGF-β signaling in the 96µCP assay, which likely contributes to lower than expected significance of ALK4/5/7 inhibitors. Lastly, in the mesendoderm responders...
we found the highest enrichment for mTOR inhibitors (p = 8.1 × 10^{-11}) (Figure 2F).

In addition to target enrichment analysis, two-dimensional hierarchical clustering (707 conditions, eight outputs, 5,656 data points) revealed several distinct phenotype clusters (Figure S3B). A cluster of compounds that enhanced PSC (blue dendrogram) also contained the CM control (Figure S3B). Similarly, a cluster that enhanced NE (red dendrogram) also contained the TiF control (Figure S3C). The few compounds that enhanced mesendoderm (purple dendrogram) clustered with two mesendoderm induction controls, BA and BA with FGF2 (BAF) (Figure 2G, right). In this cluster, 53% of the compounds targeted mTOR and no other kinase was targeted by more than one compound. Enrichment analysis of the three clusters revealed identically significantly enriched pathways as our threshold-based method (Figure S3D), further confirming the ERK/MEK and mTOR enrichments found in the screen. Based on these results we sought to further validate the ERK/MEK and mTOR results.

**ERK/MEK Inhibition Induces iPSC Differentiation to NE in a BMP-Dependent Manner**

Analyzing all the ERK/MEK inhibitors in our library, we observed a general increase in the percentage of NE cells at the expense of PSCs, with no changes in total cell number (Figures 3A and 3B). To further investigate the effect of MEK inhibition, we retested PD0325901 and found a significant reduction in PSC yield and increase in NE frequency (both p < 0.001) (Figure 3C), but found no significant increase in NE cells per colony induced relative to the blank control (p = 0.43) (Figure 3D). Reports are mixed on the effects of MEK inhibition (via PD0325901) on neural induction, and there is strong evidence that variability in endogenous factors such as BMP has hindered interpretation of these previous results (Greber et al., 2011).

To further investigate, we performed a meta-analysis of a previous dataset of the response of a panel of cell lines to a set of conditions including blank medium, PD0325901, and TiF, using the 96ωCP assay (Nazareth et al., 2013). This analysis demonstrated that PD0325901 and TiF variably induce early NE in a cell-line- and passage-dependent manner (Figure S4A). Next, to determine the dependencies of PD0325901-induced NE on other pathways, we compared the effect of PD0325901 on NE induction in the blank, with BA, or with 17 other single factors that are agonists or antagonists of major developmental pathways. When BMP4 was added with PD0325901, NE induction was reduced from 95% to less than 0.5% (p < 0.002), confirming that NE is exceptionally sensitive to BMP4, strengthening the possibility that endogenous BMP4 abolishes NE induction (Figure S4B). Notably, PD0325901 induction of NE was affected by other factors, including FGF2, activin A, TGF-β, insulin-like growth factor 1, and heregulin β1, congruent with previous proposals that inhibition of MEK, BMP, and TGF-β signaling (Greber et al., 2011) or dual BMP and TGF-β signaling (Kim et al., 2010) may indeed robustly induce NE regardless of variations in endogenous signaling. Together, these data confirm that NE induction via MEK inhibition alone is highly variable across iPSC lines, support our hypothesis that this induction is sensitive to BMP inhibition, and demonstrate that our platform can reveal subtle molecular perturbations of PSC fate.

**mTOR Inhibitors Dose-Dependently Induce Mesendoderm in iPSCs**

Our analysis indicated a strong effect of mTOR inhibition on mesendoderm induction. Conflicting reports show
that mTOR inhibition inhibits PSCs and enhances serum-mediated differentiation toward mesoderm and endoderm (Zhou et al., 2009), or may enhance the purity of pluripotent cells (Easley et al., 2010). To validate that mTOR inhibitors enhance mesendoderm induction, we screened nine mTOR inhibitors at both 1 μM and 0.1 μM, and screened eight inhibitors of additional pathways implicated in hPSC regulation. Ranking the compounds according to level of effect on mesendoderm induction (%OCT4+SOX2−), the mTOR inhibitors clearly separated from the others and the majority of conditions tested significantly enhanced the percentage of OCT4+SOX2− from the others and the majority of conditions tested with two additional replicates of rapamycin (not shown). This bimodal response to rapamycin was seen at concentrations above 0.1 nM, where mesendoderm induction peaks at 0.1 μM was the strongest responder of all the conditions, comparable with the BA control. Rapamycin binds to FKBP12 and inhibits the kinase activity of mTOR complex 1 (mTORC1), which contains mTOR and RAPTOR (Sabers et al., 1995). A separate complex containing mTOR and RICTOR (mTORC2) is thought to be rapamycin insensitive, although this is cell-line and context dependent (Lamming et al., 2012). In contrast to rapamycin and its derivatives such as temsirolimus and everolimus, which only target mTORC1, next-generation dual mTORC1/2 inhibitors have been developed, such as AZD-8055 and KU-63794. Notably, within the 18 mTOR inhibitor treatments (nine compounds at two concentrations), the top half of ranked responders are enriched for compounds only targeting mTORC1 (p < 0.005).

To further validate the effect of mTOR inhibitors on hPSCs, we performed ten-point dose curves of two mTOR inhibitors, rapamycin and AZD-8055, and again measured the four early OCT4/SOX2 subpopulations. As expected, both compounds increased the percentage of mesendoderm cells in a dose-dependent manner (Figure 4B). Surprisingly, a bimodal response to rapamycin was observed in the PSC response, where at 0.001 and 0.01 nM there was a moderate but significant enhancement of the percentage and number of PSCs. This enhancement was not seen at concentrations above 0.1 nM, where mesendoderm induction peaks at 0.1 μM (Figure 4C). The yield, and not just percentage, of PSCs are enhanced by low concentrations of rapamycin (Figure 4D). This bimodal response was seen with two additional replicates of rapamycin (not shown) but was not seen with AZD-8055, which may indicate that the mechanism is via mTORC1 inhibition (Figure 4D). Additional studies are required to understand this effect more completely.

mTOR Inhibitor-Mediated Induction of Mesendoderm Is BMP-Receptor and TGF-β-Receptor Dependent

To gain insight into the mechanism behind mTOR inhibition inducing mesendoderm in hPSCs, we screened 14 agonists and antagonists of early development signaling pathways, alone and with rapamycin (Figures 4E and S5). TGF-β1 and FGF2 moderately enhanced rapamycin-induced mesendoderm. Inhibition of either phosphatidylinositol-3-kinase (using LY-294002), a downstream effector of mTOR implicated in mesendoderm differentiation (Singh et al., 2012), or Wnt (using IWP4) did not significantly alter the effect of rapamycin. The effects of rapamycin were abolished by the BMP type 1 receptor (ALK2/3) inhibitor LDN-193189, MEK inhibitor PD-032591, and TGF-β receptor (ALK4/5/7) inhibitor SB-431542. Consistent with our results, MEK inhibition has been shown to divert differentiation from mesendoderm (Yu et al., 2011), indicating that mTOR inhibition is insufficient to overcome this effect. The LDN-193189 and SB-431542 results imply that rapamycin’s effect on mesendoderm is dependent on BMP and TGF-β receptor-mediated signaling, which are both required for hPSC differentiation toward mesendoderm (Vallier et al., 2009). Addition of either (or both) of these inhibitors to rapamycin, relative to rapamycin alone, results in loss of the OCT4+SOX2− population, no difference in the OCT4+SOX2− population, and a moderately enhanced SOX2+OCT4− population (Figure S5A), with no change in the total number of cells per colony (Figure S5B).

Based on the blocking effects of SB-431542 and LDN-193189, we reasoned that rapamycin, which induces mesendoderm alone in SF-defined medium, may act additively or synergistically with endogenous TGF-β/BMP signaling. We therefore generated a rapamycin dose curve with (rap+BA) and without (rap+BA) exogenous BMP4 (10 ng ml−1) and activin A (100 ng ml−1), and analyzed mesendoderm induction by BRACHYURY (Zhang et al., 2008) expression. The percentage and number of BRACHYURY+ cells in both rap+BA and rap+BA increased in a rapamycin dose-dependent manner. The maximum percentage of BRACHYURY+ cells achieved with rapamycin alone (19%) was comparable with the percentage achieved with BA alone (18%); however, rap+BA resulted in a more than 3-fold increased yield of BRACHYURY+ (57%) compared with BA alone (p < 0.005) (Figure 4F). The number of BRACHYURY+ cells per colony was also enhanced in rap+BA relative to BA alone by 3.8-fold (p < 0.005) (Figure 4G). Sample images are shown in Figure 4H. Similar trends were observed in dose curves generated from non-patterned hPSCs with rapamycin, or an alternative mTOR inhibitor (temsirolimus) and a BA background (Figures S5C–S5F).

Knockdown of RAPTOR Phenocopies the Mesendoderm-Inducing Effects of Rapamycin

Functionally, mTOR operates as part of two protein complexes, mTORC1 and mTORC2 (reviewed in Sabatini, 2006), which are differentially regulated by upstream
Figure 4. mTOR Inhibitors in Defined Medium Upregulate Mesendoderm via TGF-β and BMP Pathways

(A) Nine mTOR inhibitors in the OICR library were tested at 1 and 0.1 μM. Additional inhibitors of kinases implicated in early development but not targeting mTOR (“other inhibitors”) were also tested. Small molecules were ranked by effect magnitude to separate mTOR inhibitors from others. *p < 0.01, **p < 0.001 compared with blank; n = 3 independent biological replicates.

(B) Ten-point dose curves of mTOR inhibitors rapamycin and AZD-8055 show a dose-dependent increase of % mesendoderm. *p < 0.05 compared with blank control; n = 3 independent biological replicates. Blank and BA response shown as reference lines.

(legend continued on next page)
signals and have different downstream effectors. Although rapamycin was long thought to inhibit mTORC1, recent studies revealed that rapamycin can also inhibit mTORC2 in a cell-type-dependent manner (Lamming et al., 2012). To examine the role of each mTOR complex in rapamycin-induced mesendoderm differentiation, we targeted RAPTOR (mTORC1 specific) and RICTOR (mTORC2 specific) with small interfering RNA (siRNA). PSCs were seeded in pluripotency medium with siRNA against either RAPTOR (siRAPTOR) or RICTOR (siRICTOR). Two days after seeding, the medium was exchanged with fresh medium under three conditions (+blank, +rapamycin, or +BA), in the presence of siRNA. Analysis of OCT4 and SOX2 expression was conducted at day 4 (Figure 5A). In blank medium, siRAPTOR enhanced the percentage of mesendoderm (OCT4+SOX2/C0) cells relative to control scrambled siRNA (siCTRL) (p < 0.0005), but siRICTOR had no significant effect. Addition of both siRAPTOR and siRICTOR gave a phenotype similar to siRAPTOR alone, and gave rise to a significantly higher percentage of mesendoderm cells than siRICTOR alone (p < 0.05). In the presence of rapamycin, siRAPTOR again had a significant effect (C). Ten-point dose curves of rapamycin show a bimodal increase in % PSCs. *p < 0.05 compared with blank (0 rapamycin); n = 3 independent biological replicates.

(B) The effect of siRNA on BRACHYURY expression. siRNA was applied in different medium conditions as noted to non-patterned hPSCs. *p < 0.05; n = 4 independent biological replicates.

(C) Same experiment as in (B), showing number of BRACHYURY+ cells per field and total cells. **p < 0.005, ***p < 0.0005; n = 4 independent biological replicates.

(D) Images of cells stained for DNA (DAPI, blue) and BRACHYURY expression (red), with treatments as indicated. Scale bar, 500 μm. Error bars indicate SD.

(F) Dose curves of rapamycin performed alone and with BMP4 (10 ng ml⁻¹) and activin A (100 ng ml⁻¹) showing average percentage of BRACHYURY+ cells per well was quantified. *p < 0.05 and **p < 0.005 both compared with the equivalent condition without rapamycin; n = 3 independent biological replicates. Response to blank and BMP4 and activin A alone ("BA") are shown as reference lines.

(G) Same experiment as in (F) showing the number of BRACHYURY cells per colony. **p < 0.005, ***p < 0.0005 compared with the equivalent condition without rapamycin; n = 4 independent biological replicates.

(H) Images of cells stained for DNA (DAPI, blue) and BRACHYURY expression (red), with treatments as indicated. Colony outlined with dotted white line. Scale bar, 200 μm. Error bars indicate SD. See also Figure S5.
(p < 0.0005), and siRICTOR was not significantly different from siCTRL. This result indicates that rapamycin (at 0.1 μM) is not completely inhibiting mTORC1. In the presence of BA, siRAPTOR also enhances the percentage of OCT4^SOX2^ cells relative to siCTRL (p < 0.0005). Interestingly, in this medium condition, siRICTOR also enhanced the percentage of OCT4^SOX2^ (p < 0.0005), indicating that the effect of mTORC2 inhibition is dependent on TGF-β/BMP signaling. Focusing on the BA conditions, we repeated the siRNA assay and stained for BRACHYURY. As expected, siRAPTOR significantly enhanced both percentage (Figure 5B) and number (Figure 5C) of BRACHYURY^+ cells, similarly to rapamycin treatment. Sample images are shown in Figure 5D. No effect was observed from siRICTOR on BRACHYURY induction, indicating that the effect of rapamycin on BRACHYURY induction is primarily through mTORC1 and not mTORC2. These results also indicate a divergence between the OCT4^SOX2^- readout and BRACHYURY expression and further corroborate that rapamycin is acting via inhibition of mTOR signaling, not via non-specific effects.

**mTOR Inhibition Impedes Endoderm and Enhances Mesoderm Differentiation**

Based on the observation that mTOR inhibition enhances the purity and number of mesendoderm cells, we investigated the effect of rapamycin on specific downstream mesoderm and endoderm progenitors. We first differentiated hPSCs, in either BA or rap+BA (0.1 μM) for 42 hr, and assessed various markers of NE (PAX3, PAX6), mesendoderm (MIXL1, BRACHYURY), mesoderm (MEIS1, MYB), cardiac precursor (MESPI, NKX2.5, PDGFRa), hematopoietic (RUNX1), definitive endoderm (EOMES), primitive endoderm (GATA6, AFP), and trophectoderm (HAND1) lineages via qRT-PCR (Figure 6A). In general, rapamycin supplementation enhanced all differentiated lineage markers, with the exception of HAND1, a marker of trophectoderm, and EOMES, associated with definitive endoderm (DE).

We next specifically tested the effect of rapamycin on DE induction. Differentiation to DE is a multi-step process including a 1-day induction phase (D0) where cells are exposed to activin A (100 ng ml^-1) and the glycogen synthase kinase 3β (GSK3β) inhibitor CHIR99021 (CHIR, 2 μM) (Rezania et al., 2012). We tested various concentrations of rapamycin at D0 and measured the resulting FOXA2^SOX17^ DE at day 5. Although DE differentiation protocols can be extremely efficient in specific cell lines (Nostro et al., 2011), differentiation efficacy is known to vary greatly between different cell lines (Nazareth et al., 2013). Thus we tested four cell lines with varying DE induction efficiencies and demonstrated that rapamycin dose-dependently reduced DE induction (%FOX2A^SOX17^ cells) in PDX1, HES2, and HES3-RUNX1-GFP cell lines, and had no effect on H9 DE induction (Figure 6A). These results show that rapamycin does not enhance endoderm differentiation and may be specific to mesoderm induction.

Next, to test the effect of mTOR inhibition on mesoderm-derived lineages, we evaluated hematopoietic differentiation of hPSCs. During embryogenesis, hematopoietic cells are derived from specialized endothelial cells called hemogenic endothelium (HE), isolated using vascular endothelial cadherin (VECAD) and CD34 expression (Eilken et al., 2009). To track HE induction in our studies, hPSC (HES3-RUNX1-GFP cell line) aggregates were cultured in chemically defined medium (Pick et al., 2007) in the presence of rapamycin (0.1 μM) from day 0 to 2 and VECAD^CD34^ expression was assessed throughout an 8-day time course. Controls were cultured in the absence of rapamycin. The CD34^VECAD^ expression profile revealed that rapamycin addition from day 0 to day 2 enhances both purity and yield of HE (Figures 6B and 6C). Note that day-6 rapamycin-generated CD34^VECAD^ cells were also CD73^ and CD184^- (Figures 6B and 6C), further confirming their HE phenotype (Ditadi and Sturgeon, 2015). The maximum CD34^VECAD^ percentage reached in controls was 10% ± 4%, falling within the range of previously reported results in the literature (Ditadi and Sturgeon, 2015), whereas in rapamycin 30% ± 11% purity was attained (p < 0.05) (Figure 6D). Rapamycin addition also enhanced HE purity in the HES2 cell line (Figure 6D), demonstrating that our observations are not limited to the HES3-RUNX1-GFP line. Moreover, rapamycin conditions exhibited a 4-fold increase in CD34^VECAD^ cells on day 6 and a 2.5-fold increase on day 8 (99% confidence intervals shown in Figure 6E). Furthermore, kinase insert domain receptor (KDR, also known as vascular endothelial growth factor receptor 2) expression, an early marker of all blood cells (Shalaby et al., 1997), nearly doubled on day 4 in rapamycin (Figure 6E). To functionally assess whether early rapamycin treatment enhances blood progenitor induction, we seeded cells from cultures initiated either in the presence or absence of rapamycin in methylcellulose colony-forming cell (CFC) assays (Caszar et al., 2012). A 1.7-fold increase (p < 0.05) in the number of CFCs per 10^5 cells was obtained in rapamycin-treated conditions (202 ± 42 CFCs [mean ± SEM]) compared with control conditions (117 ± 26 CFCs) (Figures 6F and S6F). Additionally, gene expression analysis at days 0, 1, and 2 confirmed that rapamycin addition significantly increased the expression of BRACHYURY (p < 0.002) and MIXL1 (p < 0.0002) (Figure 6G). These results demonstrate that rapamycin treatment during days 0 to 2 of hematopoietic differentiation of hPSCs enhances the blood progenitor phenotype. A summary of our findings on the role of mTORC1 in hPSC lineage-fate decisions is presented in Figure 6G.
DISCUSSION

We have applied an engineered micro-environment based HTS platform to screen a kinase inhibitor library simultaneously for regulators of PSCs, NE, and mesendoderm. Small-molecule control of cell fate is attractive for scale-up purposes, and a focused library could be instrumental in elucidating endogenous regulators of hPSC fate decisions. In line with a previous screen (Desbordes et al., 2008), we found no single compound able to maintain long-term pluripotency, indicating that exogenous pathway agonists or possibly combinations of small-molecule inhibitors may be required for hPSC maintenance. Our multi-lineage readout enabled the discovery of specific pathways endogenously activated in hPSCs amenable to small-molecule control of lineage-specific differentiation. Patterning enabled a more rapid (48 hr versus 7 days) assay and robust response than non-pattern-based screens (Desbordes et al., 2008). Additionally, the short assay duration limits the number of emergent subpopulations, allowing simultaneous detection of these subpopulations to be tracked by OCT4 and SOX2 costaining. In conjunction with cell-number readouts, selection and induction events can be reasonably discriminated.

Our analysis revealed that mTOR inhibitors added alone to SF-defined medium can induce mesendoderm, and our follow-up studies confirmed that rapamycin enhances the formation of HE and blood progenitors. Ten-point dose curves revealed a bimodal effect where rapamycin at low concentrations mildly enhances the purity and number
of OCT4\textsuperscript{+}SOX2\textsuperscript{+} PSCs, and higher concentrations reduce the OCT4\textsuperscript{+}SOX2\textsuperscript{+} population and enhance mesendoderm differentiation. These results reconcile two previous studies: in line with Easley et al. (2010) we observe no negative effect on pluripotency of rapamycin at low concentrations, and in line with Zhou et al. (2009) we observe loss of pluripotency and induction of mesendoderm at higher concentrations. In contrast to Zhou et al., we found that rapamycin strongly inhibits DE induction. This difference may be serum mediated or cell-line dependent. Additionally, emerging evidence suggests that separate induction mechanisms may confer distinct mesoderm/endoderm subtype potentials (Mendjan et al., 2014).

Although both GSK3\textit{\beta} and mTOR inhibition appear to enhance mesendoderm, rapamycin-treated hPSCs appear to generate significantly higher frequencies of CD34\textsuperscript{+}VEcad\textsuperscript{+}CD73\textsuperscript{+} HE phenotype cells compared with ChIR99021 (GSK3\textit{\beta} inhibitor)-treated conditions. GSK3\textit{\beta} inhibition has been reported to direct hPSC differentiation to blood progenitor (Sturgeon et al., 2014) and cardiac cells (Lian et al., 2012), supporting its role as a driver of lateral plate-derived tissues. Our results indicate that mTOR inhibition is an efficient driver of hPSC differentiation to blood progenitor competent mesoderm. Comparing the mesendoderm subtypes that emerge in GSK3\textit{\beta}- and mTOR-inhibited differentiation conditions is an important future direction of our study.

The observation that subnanomolar concentrations of rapamycin have a moderate enhancing effect on pluripotency corresponds with findings of He et al. (2012) showing that rapamycin at 0.01 and 0.03 nM enhances mouse somatic cell reprogramming efficiency nearly 2-fold while concentrations of 0.05 nM or greater have no effect or reduce reprogramming efficiency. Autophagy regulates homeostasis of pluripotency-associated transcription factors including OCT4 in hPSCs, with autophagy inhibition increasing transcription factor levels yet leading to differentiation (Cho et al., 2014). Accordingly, low levels of mTOR inhibition may inhibit autophagy and enhance OCT4 levels in hPSCs sufficiently to enhance pluripotency, with higher levels of mTOR inhibition increasing OCT4 levels beyond a threshold to initiate differentiation. Alternatively, given that differentiated cell types such as hPSC-derived extra-embryonic endoderm are known to inhibit pluripotency (Peerani et al., 2007), it may be that rapamycin selectively inhibits these cell types, leading to increased hPSC numbers. Another possibility is that the cell-fate effects of discrete mTOR inhibition levels may be directly mediated by differential SMAD activation, as mTORC1 inhibition activates SMAD1 and SMAD5 in human prostate cancer cells (Wahdan-Alaswad et al., 2012), and mTORC2 inhibition can enhance SMAD2 and SMAD3 activity via regulation of the SMAD2/3-T220/T179 linker residue (Yu et al., 2015). Understanding the complex interplay between discrete levels of mTOR signaling, autophagy, pluripotency transcription factor homeostasis, signaling pathway activation, and effects on cell-fate choice and subpopulation dynamics remains an important direction of future studies.

The methods we present here are applicable to systems with heterogeneous subpopulations and complex micro-environmental regulation, such as in vitro stem cell and cancer models, and can be applied iteratively. Changes in micro-environmental cues have been attributed to cell-fate transitions (Bonfanti et al., 2010), and heterogeneity in micro-environmental context (such as population size, local cell density, and relative cell position) can deterministically explain phenotypic variance (Snijder et al., 2009). Improved techniques to control the cellular micro-environment in conjunction with improved single-cell analytics will further develop our understanding of molecular cell-fate regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

We obtained hESC lines H9 (WA09) and H7 (WA07) from the WiCell Research Institute, and HES3-RUNX1-GFP (HES3-derived RUNX1-GFP) from A. Elefanty (Monash University). All experiments were performed with H9 unless stated otherwise. These cell lines were cultured according to previously published methods (Nazareth et al., 2013). All cell stocks tested negative for mycoplasma contamination. See Supplemental Experimental Procedures for additional cell line information.

**Micro-Patterning hPSCs onto 96-Well Plates and Application of Small Molecules or Controls**

We patterned hPSCs on Matrigel into standard 96-well plates as previously described (Nazareth et al., 2013) (see Supplemental Experimental Procedures). Dissociation of hPSCs was performed using TrypLE for 3 min. TrypLE was inactivated with medium containing 20% KO-serum replacement (Invitrogen). Cells were centrifuged and resuspended in NutriStem (NS; StemGent 001-0005) and 10 μM ROCK inhibitor Y-27632. Cells were seeded at 10\(^5\) cells per well (or as described in the text) into 96-well plates, coated with either patterned or non-patterned Matrigel, and incubated for 6 hr. Cells were then washed twice with PBS and incubated for 42 hr in fresh test medium (SF supplemented with growth factors or small molecules, or CM). SF medium consists of DMEM/F12, 1× non-essential amino acids, 50 μg 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 (Seralogical), 1× trace elements A, B, and C (Mediatech), 50 μg ml\(^{-1}\) ascorbic acid (Sigma), and 7 μg ml\(^{-1}\) recombinant human insulin. The 400-compound library was obtained from the Ontario Institute of Cancer Research (OICR) (Tables S2 and S3). The following control conditions were included on each 96-well plate: blank (SF medium with no added factors), CM, B (100 ng ml\(^{-1}\) BMP4),
StemPro34 (Invitrogen), ascorbic acid (50 mM), rapamycin (0.1 mM), BA (10 ng ml⁻¹ BMP4 and 100 ng ml⁻¹ activin A), and TIF (40 ng ml⁻¹ FGF2 and 10 μM SB431542).

### Immunocytochemistry and High-Content Image Analysis

 Immunocytochemistry was performed as previously described (Nazareth et al., 2013; see also Supplemental Experimental Procedures). Single-cell x-y-coordinate and protein-expression data were exported in tab-delimited text files and imported into in-house software, ContextExplorer (Joel E.E. Ostblom, E.J.P.N., and P.W.Z., unpublished data) for exploration of colony-level details. For figures, 16-bit TIFF images were obtained for each channel, identical contrast adjustment was performed on all controls, and channels were combined into pseudo-colored composite images.

### 96-Well siRNA Transfection

Human PSCs were first seeded into non-patterned MG-treated 96-well plates at 25,000 cells per well in NS, and incubated for 48 hr. For siRNA transfection, Lipofectamine RNAiMAX transfection reagent (Invitrogen) was used as per manufacturer’s instructions, adapted for hPSCs and 96-well plates (see Supplemental Experimental Procedures).

### Viability Analysis

To assess viability, we modified the patterned hPSC assay as follows. Instead of fixing at 48 hr, calcein AM (Invitrogen) (1:1,000) and Hoechst 33342 (1:1,000) were added directly to the medium, incubated for 30 min, and then imaged.

### Differentiation of hPSCs toward Blood Progenitors and Definitive Endoderm

For ultra-high-throughput production of size-specified cell aggregates, 400-μm diameter micro-well plates (commercially available as Aggrewells, STEMCELL Technologies) were manufactured in-house in 24-well plates (Ungrin et al., 2008). For depletion of MEFs, hPSCs were dissociated with TrypLE and plated onto Geltrex (diluted 1:50) for 48 hr in NS. After 48 hr, TrypLE was added for 5 min and then quenched with 50% fetal bovine serum in DMEM/F12. The resulting single-cell suspension was seeded at 6 × 10⁵ cells per well of a micro-well plate (500 cells per aggregate) in the presence of Y-27632 (for 24 hr) at 1,500 rpm for 5 min. Aggregates were cultured in hypoxia for 8 days in base medium supplemented with cytokines BMP4 (40 ng ml⁻¹, R&D Systems), vascular endothelial growth factor (VEGF) (50 ng ml⁻¹, R&D), SCF (40 ng ml⁻¹, R&D), basic FGF (5 ng ml⁻¹, Peprotech), and rapamycin (0.1 μM, Sigma). Note that rapamycin is highly labile, and we recommend minimizing freeze-thaw cycles and titrating for maximum effect. The base medium consists of StemPro34 (Invitrogen), ascorbic acid (50 μg ml⁻¹, Sigma), L-glutamine (1% v/v, Invitrogen), penicillin/streptomycin (1% v/v, Invitrogen), 1-monoglycerol (4 × 10⁻⁴ M, Sigma), and transferrin (150 μg ml⁻¹, Roche). On day 2, hPSC-derived cells were transferred to low-cluster six-well plates, in medium without rapamycin, and cultured for an additional 6 days shaken at 85 rpm (Orbi-Shaker XL, Benchmark Scientific). Endoderm induction to FOXA2⁺SOX17⁺ DE was performed as previously described (Rezania et al., 2012).

### qRT-PCR

qRT-PCR was performed as described previously (Onishi et al., 2012). In brief, RNA was isolated using the PureLink RNA Mini Kit (Ambion) and reverse transcribed with Superscript III (Life Technologies). PCR was performed on the QuantStudio 6 Flex (Life Technologies) using SYBR Master Mix (Roche) and primers provided by the Center for Commercialization of Regenerative Medicine. Gene NCBI-RefSeq accession numbers are provided in Table S4.

### CFC Assay

A minimum of 1.5 × 10⁵ cells were seeded on day 8 in 35-mm Greiner dishes in methylcellulose-based MethoCult H4435 Enriched medium (STEMCELL). Samples were scored based on morphology 14 days after plating as described by technical manuals provided by STEMCELL.

### Statistical Analysis

Statistics were computed using one-way ANOVA, two-factor ANOVA, or linear regression as indicated. All statistics were computed in MATLAB using p values as indicated. N values are independent replicates, not technical replicates, except where noted. Enrichment analysis was performed using the hypergeometric distribution in Microsoft Excel. Z’ factor was used to assess assay quality (Zhang et al., 1999). Z’ is defined as $Z' = 1 - (3\sigma_{\text{positive}} + 3\sigma_{\text{negative}})/|\mu_{\text{positive}} - \mu_{\text{negative}}|$, where μ and σ are the mean and SD of the positive (CM-treated) and negative (BMP4-treated) control as labeled. Hierarchical clustering was performed with MeV (MultiExperiment Viewer, http://www.tm4.org/) using Euclidean distance as the similarity metric (centered) and centroid linkage as the clustering method. Exploratory analysis was performed with Microsoft Excel and Tableau (Tableau Software, www.tableau.com).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.04.003.

### AUTHOR CONTRIBUTIONS

E.N. designed, performed, and analyzed most experiments. N.R. performed all blood-induction experiments. T.Y. provided cell-culture support. M.P. and R.A. provided the kinase inhibitor library and related support. E.N. and P.W.Z. designed the project and wrote the manuscript.

### ACKNOWLEDGMENTS

This work is funded by the Canadian Institutes of Health Research (CIHR) (P.W.Z). E.J.P.N. is supported by a CIHR Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award. P.W.Z. is the Canada Research Chair in Stem Cell Bioengineering. We thank Michael Prakesh and Rima Al-awar (Ontario Institute of Cancer Research) for providing the kinase inhibitor library, Jennifer Ma for creating the graphical abstract, Celine Bauwens for...
editing assistance, and the Centre for Commercialization of Regenerative Medicine for all primers.

Received: September 3, 2015
Revised: April 4, 2016
Accepted: April 4, 2016
Published: April 28, 2016

REFERENCES

Bonfanti, P., Claudinot, S., Amici, A.W., Farley, A., Blackburn, C.C., and Barrandon, Y. (2010). Microenvironmental reprogramming of thymic epithelial cells to skin multipotent stem cells. Nature 466, 978–982.

Cai, J., Yu, C., Liu, S., Chen, S., Guo, Y., Yong, J., Lu, W., Ding, M., and Deng, H. (2009). Generation of homogeneous PDX1(+) pancreatic progenitors from human ES cell-derived endoderm cells. J. Mol. Cell Biol. 2, 50–60.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 27 (3), 275–280.

Cho, Y.H., Han, K.M., Kim, D., Lee, J., Lee, S.H., Choi, K.W., Kim, J., and Han, Y.M. (2014). Autophagy regulates homeostasis of pluripotency-associated cells in hESCs. Stem Cells 32, 424–435.

Csaszar, E., Kirouac, D.C., Yu, M., Wang, W., Qiao, W., Cooke, M.P., Boitano, A.E., Ito, C., and Zandstra, P.W. (2012). Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell Stem Cell 10, 218–229.

Desbordes, S.C., Placantonakis, D.G., Ciro, A., Socci, N.D., Lee, G., Djaballah, H., and Studer, L. (2008). High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell 2, 602–612.

Ditadi, A., and Sturgeon, C.M. (2015). Directed differentiation of definitive hemogenic endothelium and hematopoietic progenitors from human pluripotent stem cells. Methods. http://dx.doi.org/10.1016/j.ymeth.2015.10.001.

Easley, C.A.t., Ben-Yehudah, A., Redinger, C.J., Oliver, S.L., Varum, S.T., Eisinger, V.M., Carlisle, D.L., Donovan, P.J., and Schatten, G.P. (2010). mTOR-mediated activation of p70 S6K induces differentiation of pluripotent human embryonic stem cells. Cell Reprogram. 12, 263–273.

Eilkem, H.M., Nishikawa, S., and Schroeder, T. (2009). Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature 457, 896–900.

Greber, B., Coulon, P., Zhang, M., Moritz, S., Frank, S., Muller-Molina, A.J., Arauzo-Bravo, M.J., Han, D.W., Pape, H.C., and Scholer, H.R. (2011). FGF signalling inhibits neural induction in human embryonic stem cells. EMBO J. 30, 4874–4884.

Haibe-Kains, B., El-Hachem, N., Birkbak, N.J., Jin, A.C., Beck, A.H., Aerts, H.J., and Quackenbush, J. (2013). Inconsistency in large pharmacogenomic studies. Nature 504, 389–393.

He, J., Kang, L., Wu, T., Zhang, J., Wang, H., Gao, H., Zhang, Y., Huang, B., Liu, W., Kou, Z., et al. (2012). An elaborate regulation of Mammalian target of rapamycin activity is required for somatic cell reprogramming induced by defined transcription factors. Stem Cells Dev. 21, 2630–2641.

Kim, D.S., Lee, J.S., Leem, J.W., Huh, Y.J., Kim, J.Y., Kim, H.S., Park, I.H., Daley, G.Q., Hwang, D.Y., and Kim, D.W. (2010). Robust enhancement of neural differentiation from human ES and iPS cells regardless of their innate difference in differentiation propensity. Stem Cell Rev. 6, 270–281.

Lamming, D.W., Ye, L., Katajisto, P., Goncalves, M.D., Saitoh, M., Stevens, D.M., Davis, J.G., Salmon, A.B., Richardson, A., Ahima, R.S., et al. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science 335, 1638–1643.

Lian, X., Hsiao, C., Wilson, G., Zhu, K., Hazeltine, L.B., Azarin, S.M., Raval, K.K., Zhang, J., Kamp, T.J., and Palecek, S.P. (2012). Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc. Natl. Acad. Sci. USA 109, E1848–E1857.

Maherali, N., and Hochedlinger, K. (2008). Guidelines and techniques for the generation of induced pluripotent stem cells. Cell Stem Cell 3, 595–605.

Mendjan, S., Mascetti, V.L., Ortmann, D., Ortiz, M., Karjuskuaro, D.W., Ng, Y., Moreau, T., and Pedersen, R.A. (2014). NANOG and CDX2 pattern distinct subtypes of human mesoderm during exit from pluripotency. Cell Stem Cell 15, 310–325.

Nazareth, E.J., Ostblom, J.E., Lucker, P.B., Shukla, S., Alvarez, M.M., Oh, S.K., Yin, T., and Zandstra, P.W. (2013). High-throughput fingerprinting of human pluripotent stem cell fate responses and lineage bias. Nat. Methods 10, 1225–1231.

Nostro, M.C., Sarangi, F., Ogawa, S., Holtzinger, A., Corneo, B., Li, X., Micallef, S.J., Park, I.H., Basford, C., Wheeler, M.B., et al. (2011). Stage-specific signaling through TGFbeta family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development 138, 861–871.

Onishi, K., Tonge, P.D., Nagy, A., and Zandstra, P.W. (2012). Micro-environment-mediated reversion of epiblast stem cells by reactivation of repressed JAK-STAT signaling. Integr. Biol. (Camb) 4, 1367–1376.

Peerani, R., Rao, B.M., Bauwens, C., Yin, T., Wood, G.A., Nagy, A., Kumacheva, E., and Zandstra, P.W. (2007). Niche-mediated control of human embryonic stem cell self-renewal and differentiation. EMBO J. 26, 4744–4755.

Pick, M., Azzola, L., Mossman, A., Stanley, E.G., and Elefanty, A.G. (2007). Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in hematopoiesis. Stem Cells 25, 2206–2214.

Rezania, A., Bruin, J.E., Riedel, M.J., Mojibian, M., Asadi, A., Xu, J., Gauvin, R., Narayan, K., Karanu, F., O’Neill, J.J., et al. (2012). Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes 61, 2016–2029.

Sabadini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nat. Rev. Cancer 6, 729–734.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a
protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. 270, 815–822.

Shalaby, F., Ho, J., Stanford, W.L., Fischer, K.D., Schuh, A.C., Schwartz, L., Bernstein, A., and Rossant, J. (1997). A requirement for Fkh1 in primitive and definitive hematopoiesis and vasculogenesis. Cell 89, 981–990.

Singh, A.M., Reynolds, D., Cliff, T., Ohtsuka, S., Mattheyes, A.L., Sun, Y., Menendez, L., Kulik, M., and Dalton, S. (2012). Signaling network crosstalk in human pluripotent cells: a Smad2/3-regulated switch that controls the balance between self-renewal and differentiation. Cell Stem Cell 10, 312–326.

Smith, J.R., Vallier, L., Lupo, G., Alexander, M., Harris, W.A., and Pedersen, R.A. (2008). Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neuroectoderm. Dev. Biol. 313, 107–117.

Snijder, B., Sacher, R., Ramo, P., Damm, E.M., Liberali, P., and Pelkmans, L. (2009). Population context determines cell-to-cell variability in endocytosis and virus infection. Nature 461, 520–523.

Snijder, B., Sacher, R., Ramo, P., Liberi, P., Mench, K., Wolfrum, N., Burleigh, L., Scott, C.C., Verheije, M.H., Mercer, J., et al. (2012). Single-cell analysis of population context advances RNAi screening at multiple levels. Mol. Syst. Biol. 8, 579.

Sturgeon, C.M., Ditadi, A., Awong, G., Kennedy, M., and Keller, G. (2014). Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. Nat. Biotechnol. 32, 554–561.

Sun, N., Yazawa, M., Liu, J., Han, L., Sanchez-Freire, V., Abilez, O.J., Navarrete, E.G., Hu, S., Wang, L., Lee, A., et al. (2012). Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. Sci. Transl Med. 4, 130ra147.

Tsutsui, H., Valamehr, B., Hindoyan, A., Qiao, R., Ding, X., Guo, S., Witte, O.N., Liu, X., Hu, C.M., and Wu, H. (2011). An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. Nat. Commun. 2, 167.

Ugrin, M.D., Joshi, C., Nica, A., Bauwens, C., and Zandstra, P.W. (2008). Reproducible, ultra high-throughput formation of multi-cellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One 3, e1565.

Vallier, L., Reynolds, D., and Pedersen, R.A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. 275, 403–421.

Vallier, L., Alexander, M., and Pedersen, R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. 118, 4495–4509.

Vallier, L., Touboul, T., Brown, S., Cho, C., Bilican, B., Alexander, M., Cedervall, J., Chandran, S., Ahrlund-Richter, L., Weber, A., et al. (2009). Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. Stem Cells 27, 2655–2666.

Wahdan-Alaswad, R.S., Bane, K.L., Song, K., Shola, D.T., Garcia, J.A., and Danielpour, D. (2012). Inhibition of mTORC1 kinase activates Smads 1 and 5 but not Smad8 in human prostate cancer cells, mediating cytostatic response to rapamycin. Mol. Cancer Res. 10, 821–833.

Yu, P., Pan, G., Yu, J., and Thomson, J.A. (2011). FGF2 sustains NANOG and switches the outcome of BMP4-induced human embryonic stem cell differentiation. Cell Stem Cell 8, 326–334.

Yu, J.S., Ramasamy, T.S., Murphy, N., Holt, M.K., Czapiewski, R., Wei, S.K., and Cui, W. (2015). PI3K/mTORC2 regulates TGF-beta/Activin signalling by modulating Smad2/3 activity via linker phosphorylation. Nat. Commun. 6, 7212.
Supplemental Information

A Multi-Lineage Screen Reveals mTORC1 Inhibition Enhances Human Pluripotent Stem Cell Mesendoderm and Blood Progenitor Production

Emanuel Joseph Paul Nazareth, Nafees Rahman, Ting Yin, and Peter William Zandstra
Figure S1

**Panel a**

![Graph showing cell differentiation percentages.](image)

**Panel b**

|         | H\(_{\text{CM}}\) | σ\(_{\text{CM}}\) | H\(_{\text{BMP}}\) | σ\(_{\text{BMP}}\) | Z'   |
|---------|------------------|------------------|-------------------|------------------|------|
| Plate 1 | 93.6             | 4.0              | 0.8               | 1.5              | 0.82 |
| Plate 2 | 94.2             | 0.8              | 0.1               | 0.1              | 0.97 |
| Plate 3 | 87.7             | 0.0              | 0.3               | 0.4              | 0.99 |
| Plate 4 | 98.3             | 0.7              | 0.0               | 0.0              | 0.98 |

**Panel c**

|         | H\(_{\text{FGF2}}\) | σ\(_{\text{FGF2}}\) | H\(_{\text{BMP}}\) | σ\(_{\text{BMP}}\) | Z'   |
|---------|---------------------|---------------------|-------------------|------------------|------|
| Plate 1 | 1745                | 359                 | 251               | 47               | 0.18 |
| Plate 2 | 1772                | 237                 | 246               | 42               | 0.45 |
| Plate 3 | 1458                | 265                 | 247               | 45               | 0.23 |

Non-patterned assay (Desbordes et al., OCT4 signal intensity [A.U.])
Figure S3

Inhibition enhances pluripotency

Inhibition enhances neuroectoderm

Inhibition enhances primitive streak

-log(p-value)

-Log(p-value)

-Log(p-value)
Figure S4

a

b
Figure S6

(a) Log2(Fold change in transcript relative to NS)

(b) +blank +BA +CHIR99021 +rapamycin

(c) CD34+VECAD+

(d) CD34+VECAD+ (%)

(e) KDR+ (%)

(f) CFCs per 10^5 cells

(g) Relative log2 fold change in transcript relative to D0
Figure S1 Screen characterization, related to Figure 1. (A) Response of control conditions, distributed across 10 96-well plates. N= independent biological replicates as indicated. (B) Z’ analysis using positive and negative controls of patterned and non-patterned hPSC assays. Non-patterned data from Desbordes et al. (C) Z’ comparison of non-patterned and patterned assays. N=4 plates for patterned, N=3 plates for non-patterned. Error bars indicate standard deviation. * indicates $p < 0.01$.

Figure S2 Responding compounds, related to Figure 2. Oct4/Sox2 percentages and numbers per colony were obtained for each unique treatment, and treatment with base medium alone was subtracted. Select treatments are labeled with the compound name. (A) Responding pluripotency enhancing compounds. Arrow indicates CM control condition. (B) Responding neuroectoderm enhancing compounds. Arrow indicates TiF control condition. (C) Responding primitive streak enhancing compounds. Arrow indicates BA control condition, and BA with FGF2 (“BAF”).

Figure S3 Target enrichment in clusters, related to Figure 2. (A) Kinase inhibitor library count of unique compounds targeting each kinase target. Kinases with one targeting compound not shown. (B) Enhanced pluripotency cluster from two-dimensional hierarchical clustering, detailed view, indicating compound name, concentration. (C) Enhanced early neuroectoderm cluster detailed view, indicating compound name, concentration. (D) Enrichment in pluripotency, neuroectoderm, and in primitive streak clusters obtained through two-dimensional hierarchical clustering. $p$-values were obtained for each target, using the hypergeometric distribution, and shown in $-\log_{10}$ base 10. Threshold corresponds to $p = 0.001$.

Figure S4 MEK inhibitor PD0325901 induced neuroectoderm is sensitive to BMP inhibition but not Activin A inhibition, related to Figure 3. (A) Cell line comparison of neuroectoderm induction using base medium alone (“blank”), TGFβ inhibitor and FGF2 (TiF), and PD0325901. N=3 independent biological replicates. Meta-analysis of data provided in Nazareth et. al Nature Methods 2013. (B) 19 conditions were screened alone or with added PD0325901, and neuroectoderm induction response was measured to evaluate interactions. PD0325901 induction of neuroectoderm is insensitive to all factors measured except BMP4. * indicate $p < 0.002$. N=12 independent biological replicates for “+blank+blank” condition, all other conditions N=2 independent biological replicates if error bars shown, N=1 well if no error bars shown. Error bars indicate standard deviation.

Figure S5 Mechanism and validation studies on mTOR inhibition mediated induction of mesendoderm, related to Figure 4. (A) TGFβ1 moderately but not statistically significantly enhances the mesendoderm inducing effect of rapamycin, while SB-431542 (an inhibitor of TGFB type I receptor ALK5) and LDN-193189 (an inhibitor of BMP type I receptors ALK2 and ALK3) abolish the mesendoderm-inducing effect of rapamycin. * indicates $p < 0.02$. N=3. (B) TGFβ1, SB-431542, and LDN-193189 do not affect total cell number per colony when added with rapamycin. N=3 independent biological replicates. (C) Dose curve of rapamycin in the presence of BMP4 (10 ng/ml) and Activin A (100 ng/ml), applied to non-patterned hPSCs. Resulting Brachyury expression was quantified. Average percentage of Brachyury cells per well is shown. BMP4 and Activin A alone shown as reference line. N=3 independent biological replicates. (D) Same experiment, showing number of Brachyury+ cells per well. N=3 independent biological replicates. (E) Dose curve of temsirolimus in the presence of BMP4 (10 ng/ml) and Activin A (100 ng/ml), applied to non-patterned hPSCs. Resulting Oct4 and Sox2 expression was quantified. Average percentage of
Oct4+Sox2- cells per well is shown. N=3 independent biological replicates. (F) Same experiment, showing number of Oct4+Sox2- cells per well. N=3 independent biological replicates. All error bars indicate standard deviation.

**Figure S6** mTOR inhibitor effects on endoderm and blood induction, related to Figure 6. (A) qRT-PCR on hPSCs exposed to BA or BA+rapamycin (0.1 µM) for 42 h. Data is presented as mean, error bars indicated standard deviation. N=3 independent biological replicates. (B) Representative flow cytometry plots from day 6 showing (top) CD34/VECAD induction and (bottom) CD184/CD73 induction in control (+blank), control + BMP4 (10 ng/ml)+Activin A (100 ng/ml) (+BA), control + CHIR99021 (3 µM) and control + rapamycin treatments. (C) Fold change of CD34+VECAD+ and CD34+VECAD+CD73- cellular yield in +blank, +BA, +CHIR99021, and +rapamycin treatments at day 6 (N=3, independent biological replicates). ** indicates p < 0.01 (Student's t-Test). (D) Differentiation of HES2 cell line towards hemogenic endothelium is enhanced by rapamycin. Comparison of the VECAD+CD34+ percentage obtained with or without rapamycin. N=4 independent biological replicates. Error bars indicate standard deviation. ** indicates p < 0.01. (E) Differentiation of HES3-RUNX1-GFP cell line towards hemogenic endothelium is enhanced by rapamycin. Time course showing KDR+ cells throughout day 1-8 of differentiation showing percentage (left) and numbers per well (right) with and without rapamycin treatment. (F) CFC comparison of definitive blood obtained with and without rapamycin. N=3, independent experiments of the HES2 (one replicate) and RUNX1-GFP (two replicates) cell lines. ‡ indicates p<0.05 (paired Student's t-Test). (G) Gene expression time-course with rapamycin, relative to control. Log2(Fold change in transcript relative to D0) was determined during day 0, 1, and 2 of the blood induction protocol for SOX2, T, MIXL1, MESP1, and FOXA2, both during the standard protocol and when rapamycin (0.01 µM) was added. Control values were subtracted from the +rapamycin values to obtain the relative Log2(Fold change in transcript relative to D0). qPCR samples were performed with N=3 technical replicates.
### Enrichment analysis - Pluripotency responders

| Kinase target | Count in responders | Count in screen | p value |
|---------------|---------------------|-----------------|---------|
| VEGFR         | 3                   | 34              | 0.06316 |
| Akt           | 2                   | 19              | 0.0935  |
| JAK           | 2                   | 22              | 0.11581 |
| DNA-PK        | 1                   | 5               | 0.13533 |
| CDK           | 2                   | 35              | 0.20397 |

### Enrichment analysis - Neuroectoderm responders

| Kinase target | Count in responders | Count in screen | p value     |
|---------------|---------------------|-----------------|------------|
| MAPK          | 11                  | 37              | 2.9215E-05 |
| ALK4/5/7      | 5                   | 12              | 0.0010     |
| v-Src         | 2                   | 2               | 0.0057     |
| Diacylglycerol (DAG) Kinase inhibitor | 2 | 4 | 0.0294 |
| ErbB2/ErbB4   | 2                   | 7               | 0.0817     |

### Enrichment analysis - Primitive streak responders

| Kinase | Count in hits | Count in screen | p value     |
|--------|---------------|-----------------|------------|
| mTor   | 8             | 17              | 2.9911E-10 |
| SMG-1  | 1             | 1               | 0.0235     |
| SRC    | 1             | 6               | 0.1262     |
| cKIT   | 1             | 6               | 0.1262     |
| GSK-3  | 1             | 14              | 0.2456     |
## Table S4 qRT-PCR genes and NCBI-GenBank accession numbers

| Lineage                  | Official Symbol | RefSeq ACCESSION |
|--------------------------|-----------------|------------------|
| Primitive Ectoderm       | GATA6           | NM_005257.4      |
| Primitive Streak         | MIXL1           | NM_031944.1      |
| Primitive Streak         | T               | NM_003181.2      |
| Mesoderm                 | MEIS1           | NM_002398.2      |
| Mesoderm                 | MYB             | NM_001130173.1   |
| Definitive Endoderm      | EOMES           | NM_005442.2      |
| Neuroectoderm            | PAX6            | NM_000280.4      |
| Neural crest             | PAX3            | NM_181457.3      |
| Primitive endoderm       | AFP             | NM_001134        |
| Trophectoderm            | HAND1           | NM_004821.2      |
| Cardiac precursor        | MESP1           | NM_018670.3      |
| Cardiac precursor        | NKX2-5          | NM_004387.3      |
| Cardiac precursor        | PDGFRA          | NM_006206.4      |
| Hematopoietic            | RUNX1           | NM_001754.4      |
| Reference                | GAPDH           | NM_002046.3      |
| Reference                | GUSB            | NM_000181.1      |
Experimental Procedures

Cell culture

ZAN3 and ZAN11 hiPSC lines were derived from activated CD3+ T cells enriched from umbilical cord blood (Nazareth et al., 2013). HES2 (hESC) was provided by G. Keller (McEwen Centre for Regenerative Medicine/University Health Network). BJ1D (hiPSC) was provided by M. Radisic (University of Toronto). CA1 (hESC) was provided by A. Nagy (Samuel Lunenfeld Research Institute). R306C (Hotta et al., 2009), RTT-Δ3-4 #37, and T158M #5 (ref. (Cheung et al., 2011)), all Rett syndrome hiPSC disease models, as well as BJ4YA (hiPSC) were provided by J. Ellis (The Hospital for Sick Children). ZAN3, ZAN11, H9 and H7 were routinely cultured on feeder layers of irradiated MEFs in knockout (KO)-Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 20 % KO-serum replacement (Invitrogen) and supplemented with 4 ng mL\(^{-1}\) FGF-2 (PeproTech) (KO-DMEM). Cells were passaged 1:4-1:6 every 4-5 days, and were disassociated into small clumps using 0.1 % collagenase IV (Invitrogen).

HES2 and HES3-RUNX1-GFP were cultured on growth factor reduced Matrigel\textsuperscript{TM} (MG) in KO-DMEM based media supplemented with 20 ng mL\(^{-1}\) FGF-2 (PeproTech), and passaged every 4-5 days using TrypLE Express (Invitrogen). CA1 was cultured on MG using NS as per manufacturer’s instructions. BJ1D, R306C, RTT-Δ3-4 #37, T158M #5, and BJ4YA were cultured on MG using mTeSR1 (STEMCELL Technologies) as per manufacturer’s instructions.

Microcontact printing of substrate into 96-well plates

We have previously developed a method for patterning proteins in standard 96-well plates (Nazareth et al., 2013). In brief, polydimethylsiloxane (PDMS) stamps were fabricated using standard soft lithography techniques, with the exception that liquid PDMS was cast in a Teflon mould before curing, allowing control of the shape of the PDMS stamp. PDMS stamps were cast to fit directly into 96-well plates. ECM solution consisted of 0.00125 % fibronectin (Sigma-Aldrich, F1141) and 0.002 % gelatin (Sigma-Aldrich, G9391) in phosphate buffered saline (PBS). ECM solution was deposited onto the patterned surface of ethanol sterilized PDMS stamps for 4h at room temperature. Stamps were rinsed with ddH2O, dried gently with N2 gas, placed into tissue-culture treated 96-well plates (Costar) ensuring conformal contact between the plate and PDMS stamp, and 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.

96-well siRNA transfection

HPSCs were first seeded into MG treated 96-well plates (non-patterned) at 25,000 cells per well in NS, and incubated for 48 h. For siRNA transfection in 96-well plates, 0.15 µl per well Lipofectamine\textsuperscript{®} RNAiMAX Transfection Reagent (Invitrogen, cat. no. 13778030) and 20 µl per well Opti-MEM\textsuperscript{®} (Invitrogen, cat. no. 31985062) were first mixed and incubated for 5 min at room temperature. Next, 20 µl per well of this solution was transferred to a V-bottom 96-well plate. 0.3 µl per well siRNA (at 20 pm per µl) was then added to each well, mixed, and incubated for an additional 20 min at room temperature ("siRNA mix"). 48 h after hPSCs were seeded into 96-well plates, the medium was removed and 20 µl per well of siRNA mix was added along with 40 µl per well fresh NS. After 6 h of incubation (T=54h) 160 µl of NS was added per well, and the hPSCs were cultured for an additional 42 h. On day 4,
medium was removed and 20 µl per well of siRNA mix was added (with wells treated with the same siRNA on day 2 and day 4) along with 40 µl per well of treatment media (SF+blank, SF+rapamycin, SF +BA, or NS ). 6 h later 160 µl of the same treatment media was added to each well, and cells were incubated for an additional 42 h before fixing.

**Immunocytochemistry and high-content image analysis**

Plates were fixed for 30 min in 3.7 % formaldehyde and permeabilized for 3 min in 100 % methanol. Plates were imaged and analyzed using the Cellomics Arrayscan VTI platform and Target Activation algorithm (Thermo Scientific). This algorithm generates nuclear masks, provides single cell nuclear intensity values for protein expression (OCT4, SOX2 etc.), DNA content through Hoechst staining, as well as spatial x- and y-coordinates of the nuclei centroids. Fluorescent images were obtained of OCT4 (1:500; BD, BD611203), SOX2 (1:500; R&D Systems, MAB2018), BRACHYURY (1:200, R&D Systems, AF2085), FOXA2 (1:500, Abcam, ab40874), SOX17 (1:500, R&D, AF1924) and Hoechst 33342 (Sigma-Aldrich, B2261). Primary antibodies were incubated overnight in 10 % FBS in PBS at 4° C. AlexaFluor secondary antibodies (1:500; Molecular Probes) were incubated for 1 h in 10% FBS in PBS at room temperature.