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Brief Report: Cell Cycle Dynamics of Human Pluripotent Stem Cells Primed for Differentiation

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ABSTRACT

Understanding the molecular properties of the cell cycle of human pluripotent stem cells (hPSCs) is critical for effectively promoting differentiation. Here, we use the Fluorescence Ubiquitin Cell Cycle Indicator system adapted into hPSCs and perform RNA sequencing on cell cycle sorted hPSCs primed and unprimed for differentiation. Gene expression patterns of signaling factors and developmental regulators change in a cell cycle-specific manner in cells primed for differentiation without altering genes associated with pluripotency. Furthermore, we identify an important role for PI3K signaling in regulating the early transitory states of hPSCs toward differentiation. STEM CELLS *2019;37:1151–1157*

SIGNIFICANCE STATEMENT

Generating differentiated cell types from human pluripotent stem cells (hPSCs) holds great therapeutic promise but has proven to be challenging in practice. The cell cycle may play an important role in enhancing the differentiation potential of hPSCs. Here, the authors track and isolate hPSCs from different phases of the cell cycle and perform RNA sequencing. The data show that gene expression patterns of signaling factors and developmental regulators change in a cell cycle-specific manner as hPSCs transition toward differentiation and highlight an important role for PI3K signaling in regulating these early transitory states.

INTRODUCTION

Despite recent advances in generating specialized cell types from human pluripotent stem cells (hPSCs), many studies have noted that pluripotent stem cell lines often have an inherent inability to differentiate even when stimulated with a proper set of signals [1–5]. The cell cycle, particularly the G1 phase, may play an important role in enhancing the differentiation potential of PSCs [2, 6–9]. However, simply lengthening the G1 phase in embryonic stem cells is not sufficient to facilitate differentiation [10], suggesting that an improved understanding of the molecular properties of the embryonic cell cycle is needed.

In a prior study, we demonstrated that transiently treating hPSCs with dimethylsulfoxide (DMSO) for 24 hours prior to directed differentiation significantly increases the propensity for differentiation across all germ layers [2, 11]. This technique is now used by multiple laboratories to improve differentiation across species (including mouse, rabbit, primate, and human) into more than a dozen lineages, ranging from neurons and cortical spheroids to smooth muscle cells to hepatocytes [12–15]. Although the DMSO treatment activates the retinoblastoma protein (Rb) and increases the percentage of hPSCs in the G1 phase of the cell cycle [2, 11], it remains unknown whether the DMSO treatment simply enriches cells in G1 or whether there are intrinsic changes to the cell cycle following the DMSO treatment that may potentiate differentiation.

Here, we use Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) technology to systematically track and understand cell division in hPSCs primed and unprimed for differentiation [16]. The FUCCI system fuses red-emitting and green emitting fluorescent proteins to the cell cycle ubiquitination oscillators, Cdt1 and Geminin, whereby Cdt1 tagged with red fluorescence is present only when cells are in G1 and geminin tagged with green fluorescence is only present when cells reside in the S/G2/M phases. By performing RNA-sequencing on hPSCs sorted from the early G1, late G1, and SG2M phases of the cell cycle, we show that gene expression patterns of signaling factors and developmental regulators change in a cell cycle-specific manner in cells primed for differentiation following a 24 hours DMSO treatment. Changes in signaling pathways controlling cell proliferation, differentiation, and apoptosis, particularly the phosphoinositide 3-kinase (PI3K) pathway, were regulated by the DMSO treatment. Concordantly, transiently inhibiting PI3K signaling enhances hPSC differentiation across all germ layers.

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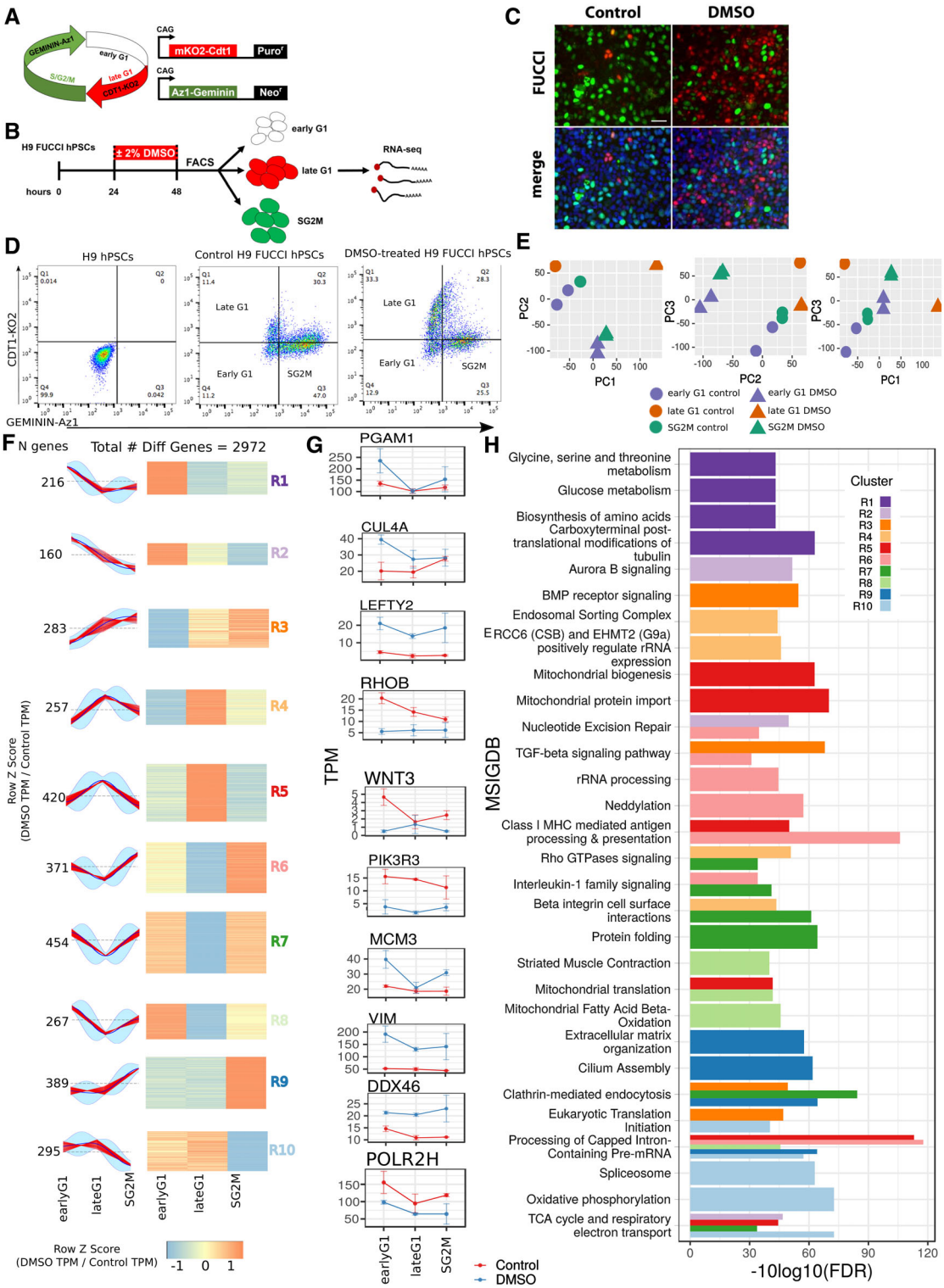

Figure 1. Dimethylsulfoxide (DMSO) treatment of human pluripotent stem cells (hPSCs) changes gene expression trajectories in response to phase of the cell cycle. **(A):** Schematic representation of the Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) technology labeling individual late G1 phase nuclei in red and S/G2/M phase nuclei in green, whereas early G1 phase nuclei are double negative. **(B):** Schematic of H9-FUCCI hPSCs treated with or without 2% DMSO for 24 hours followed by cell cycle sorting and high throughput RNA-sequencing. **(C):** Immunofluorescent images of control and DMSO-treated H9 FUCCI hPSCs in the late G1 phase (red) and SG2M phases (green) of the cell cycle. Scale bar: 35 μm. **(D):** Fluorescence activated cell sorting of cells in the early G1 (double negative), late G1 (red), and SG2M (green) phases of the cell cycle in control and DMSO-treated FUCCI hPSCs. **(E):** Principal component analysis of batch-corrected RNA-seq expression data. PC1 (97.02% variance explained) versus PC3 (0.38% variance explained) are plotted on transcripts per million (TPM) data for hg19. **(F):** Dirichlet process Gaussian process (DPGP) mixture model clustering of differentially expressed genes. DPGP

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To the best of our knowledge, this is the first study to systematically perform RNA-seq on cell cycle sorted populations of hPSCs to investigate changes that occur within a cell line as cells transition toward a state for differentiation. This comprehensive analysis begins to shed light on important signaling pathways, particularly PI3K, in regulating the developmental potential of hPSCs during early transitory states.

Materials and Methods

Detailed methods are shown in Supporting Information online.

Results

Gene Expression Dynamics Associated with Cell Cycle Progression in hPSCs

To begin, we use the FUCCI system adapted into the hPSC H9 cell line [7] to systematically track and isolate hPSCs from different phases of the cell cycle (Fig. 1A). H9 FUCCI hPSCs were cultured under maintenance conditions in mTESR (control) or with 2% DMSO for 24 hours to prime hPSCs for differentiation (Fig. 1B). Following treatment with DMSO, there is a shift in cells from SG2M to G1 (Fig. 1C, 1D). We next used fluorescence-activated cell sorting to isolate cells from early G1, late G1, and SG2M phases from control and 24 hours DMSO-treated H9 FUCCI hPSCs (Fig. 1B, 1D) and performed RNA-sequencing. Using principal component analysis, we found that the strongest source of variation was in treatment versus control (PC1), followed by phase of the cell cycle (PC2; Fig. 1E). We next used the nonparametric Dirichlet process Gaussian process mixture model [17] to cluster fold changes in aligned reads (normalized to transcripts per million [TPM]) to assess changes in gene expression patterns associated with cell cycle progression. A total of 2,972 differentially expressed genes (false discovery rate (FDR) < 0.05) underwent clustering and 10 clusters emerged (Fig. 1F) with genes upregulated or downregulated in response to phase of the cell cycle following the DMSO treatment. The largest clusters consisted of genes with decreased expression in late G1 but high in early G1 and SG2M (cluster 7 with 454 genes) or increased expression in late G1 and reduced in early G1 and SG2M (cluster 5 with 420 genes) following the DMSO treatment. Genes with trajectories characteristic of the 10 clusters include those playing important roles in early development and regulating growth signaling pathways (e.g., phosphoglycerate mutase 1, left-right determination factor 2, ras homolog family member B, wingless-type family member 3, phosphoinositide-3-kinase regulatory subunit 3), ubiquitination and DNA repair (cullin 4A), DNA replication licensing (e.g., minichromosome maintenance complex component 3), maintaining cell shape and cytoskeletal interactions (e.g., vimentin, RHOB), and regulating transcription, splicing, and translation of genes through critical RNA helicases and polymerases (e.g., DDX46, POLR2H; Fig. 1G). Annotation of the gene sets representative of each cluster using the Molecular Signatures Database (MSigDB) shows the most significant pathways enriched in the 10 clusters (Fig. 1H). Across all clusters, the DMSO treatment targeted pathways known to be tightly coordinated with the cell cycle, playing critical roles in cytoskeletal organization and membrane structure, transcriptional regulation, cell growth control, and development (e.g., Rho GTPases signaling, mitochondrial biogenesis, rRNA processing, neddylation, protein folding, extracellular matrix organization, cilium assembly, pre-mRNA processing, spliceosome; Fig. 1H). Genes associated with 5 of the 10 clusters (R5, R6, R8, R9, and R10) were enriched in the processing of capped intron-containing pre-mRNA (Fig. 1H, indicating an important role for the DMSO treatment in regulating the efficiency and fidelity of gene expression [18]. Many pathways associated with mitochondrial function were also enriched (clusters R5 and R8), consistent with recent work demonstrating that mitochondrial dynamics play critical roles in the developmental potential of hPSCs [19]. Overall, this data illustrates that the DMSO treatment changes the expression of these genes in a phase-specific manner in hPSCs, and thereby restricts their activity in a temporal manner that is otherwise not present in the cell cycle of untreated control hPSCs.

Regulatory Role for PI3K-AKT Signaling in hPSC Differentiation

In aggregate, UpSet analysis shows that the most number of differentially expressed genes occur in the late G1 phase and are specific to distinct phases of the cell cycle—of the 1,078 genes downregulated in late G1, 783 were not significantly altered at the other cell cycle phases; of the 895 upregulated genes, 554 were unique to late G1 (Fig. 2A). MSigDB pathway analysis (FDR < 0.01) shows that DMSO affects a number of pathways associated with cell signaling (Fig. 2B). Across all of the signaling pathways targeted by DMSO, PI3K was the most commonly represented gene followed by PIK3CA, a catalytic subunit of PI3K (Fig. 2B). Kyoto Encyclopaedia of Genes and Genomes analysis shows that 48 genes associated with the PI3K-AKT pathway are significantly regulated by the DMSO treatment at one or more phases of the cell cycle (Fig. 2C, Supporting Information Fig. S1). Many genes upstream in the pathway (e.g., PI3K receptors, PI3K, Ras) are generally downregulated in the early and late G1 phases of the cell cycle. Other signaling pathways regulated by DMSO also converge upon PI3K and PI3KR signaling (examples illustrated in Supporting Information Figs. S2–S4), a pathway well known to regulate cell cycle, proliferation, differentiation, apoptosis, and growth and metabolism [20–24].

Concordantly, pathways and genes associated with mitosis and the cell cycle (e.g., cell cycle checkpoints, p value = 3.98e−8) were also significantly regulated by the DMSO treatment through MSigDB pathway and gene ontology (GO) enrichment

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clustering was applied to the fold change of (TPM DMSO/TPM control) for differential genes and yielded 10 clusters, labeled R1–R10. The z-scores for genes in each cluster are plotted in heatmap form as well as line plots of trajectories across early G1, late G1, and SG2M. Red lines indicate fold change trajectories for individual peaks assigned to the cluster. The light blue cloud indicates values within 2 SD of the cluster mean. **(G):** Representative differentially expressed genes for each DPGP cluster R1–R10. TPM values with SD are indicated for control (red) and DMSO (blue) at the early G1, late G1, and SG2M phases. **(H):** Most significant Molecular Signatures Database pathways enriched in the DGPGP clusters R1–R10. Height of the bar indicates −log10(FDR) values for the corresponding clusters.

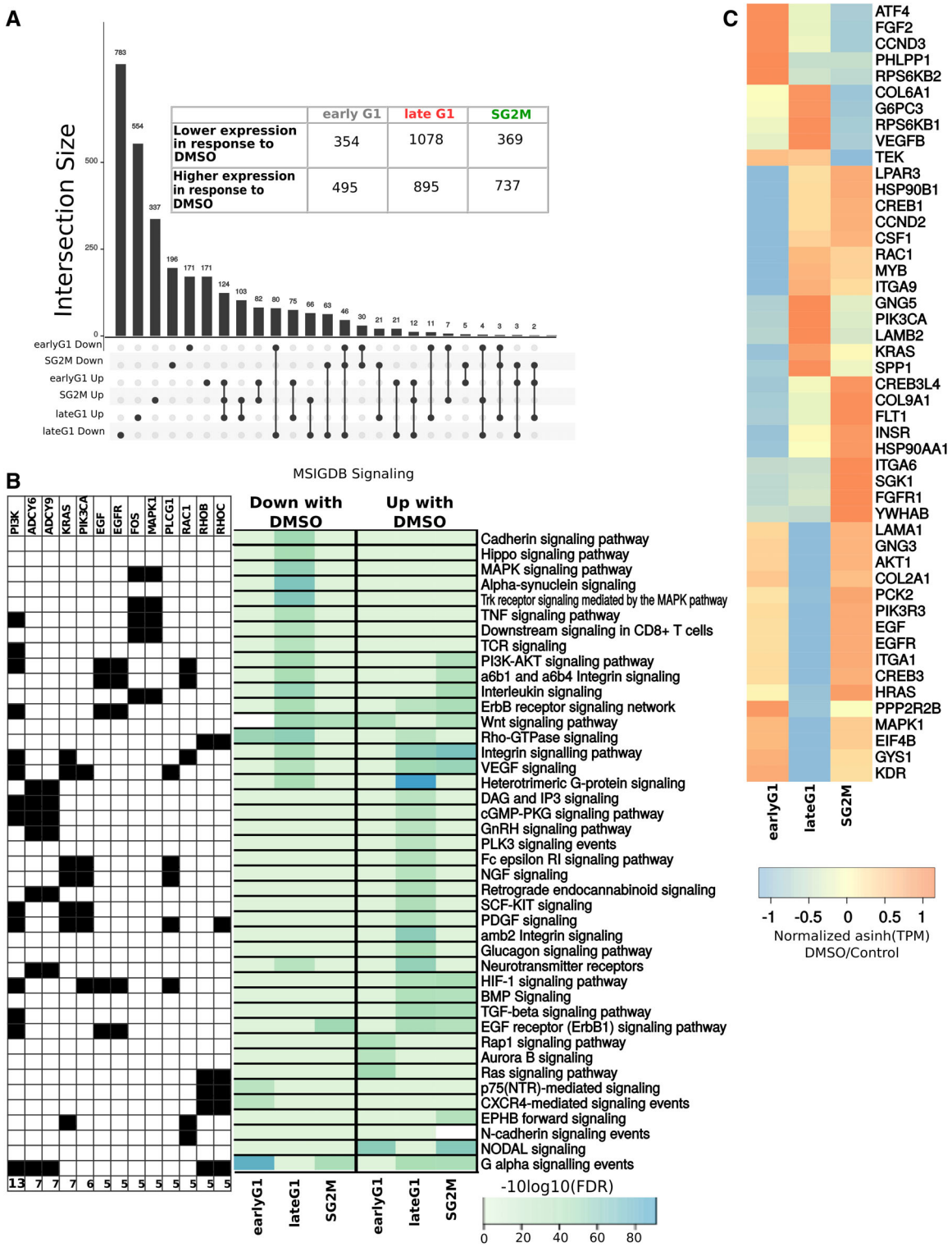

Figure 2. Dimethylsulfoxide (DMSO)-induced changes converge upon PI3K signaling. **(A):** Number of differentially expressed genes (FDR < 0.05, LFC ≥ 1) in control versus DMSO-treated Fluorescence Ubiquitin Cell Cycle Indicator human pluripotent stem cells in the early G1, late G1, and SG2M phases. UpSetR diagram of differentially expressed genes shows that number of differential genes that increase (up) or decrease (down) in expression in response to DMSO at the early G1, late G1, and/or SG2M phases. **(B):** Enriched REACTOME pathways for differential genes at the early G1, late G1, and SG2M phases of the cell cycle. The heatmap shading corresponds to the −10log10(FDR) for each pathway across the different phases of the cell cycle. Genes with differential expression in response to DMSO treatment that are present in five or more differential signaling pathways are indicated with black boxes in the grid to the left of the heatmap. **(C):** Differentially expressed genes within the PI3K-AKT signaling pathway. Heatmap values are row *z*-scores of asinh(transcripts per million [TPM]) DMSO/asinh(TPM) controls.

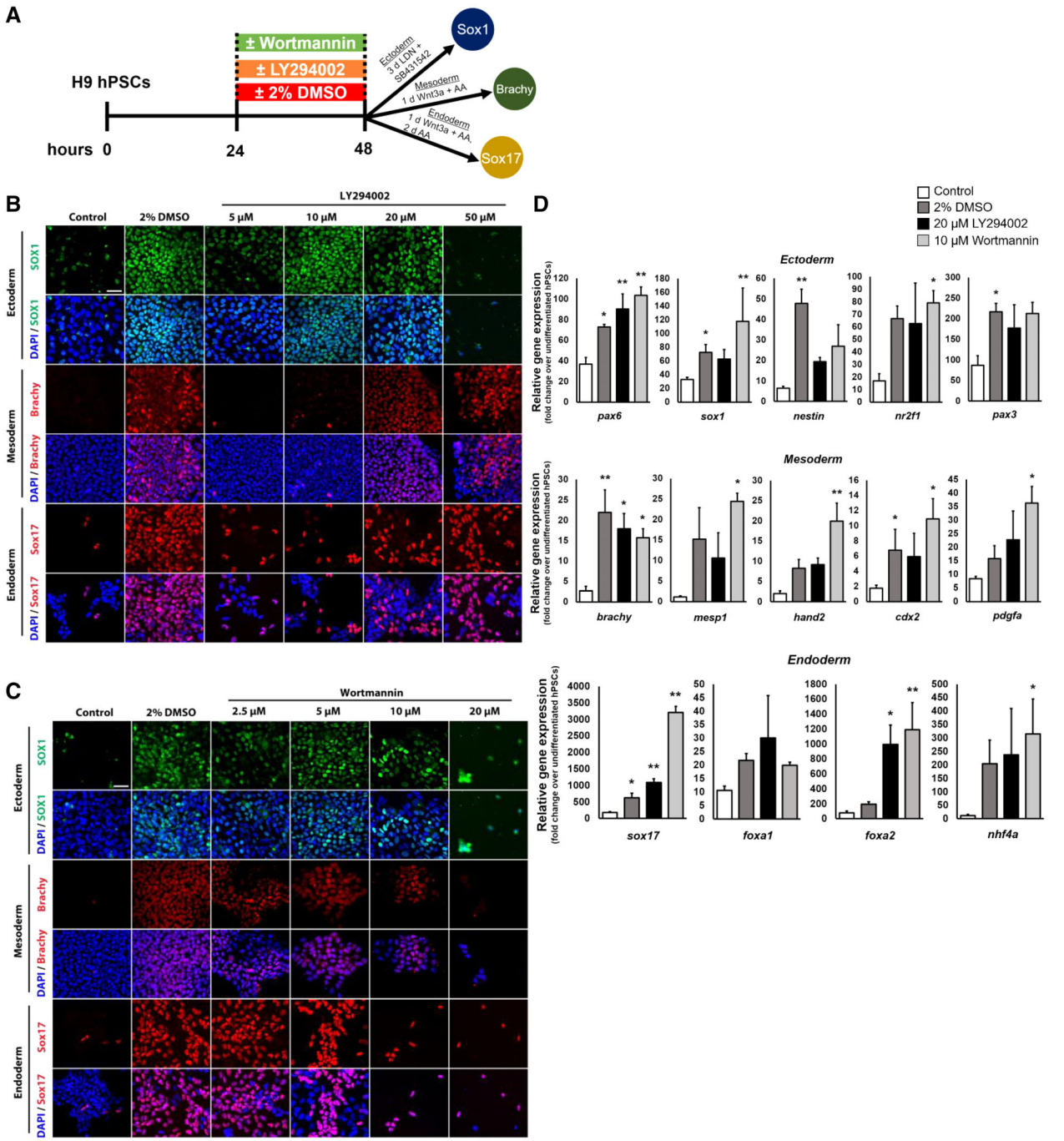

Figure 3. PI3K inhibition increases human pluripotent stem cell (hPSC) differentiation across all germ layers. **(A):** Schematic of H9 hPSCs treated with 2% dimethylsulfoxide (DMSO) or inhibitors of PI3K (LY294002 or Wortmannin) for 24 hours and subsequently directly differentiated into the ectodermal, mesodermal, and endodermal germ layers. Immunostaining for germ layer specific markers following treatment with **(B)** LY294002 or **(C)** Wortmannin compared with untreated control and 2% DMSO-treated hPSCs. **(D):** Quantitative RT-PCR for lineage-specific genes following directed differentiation of LY294002 (20 µM) or Wortmannin (10 µM) treated hPSCs compared with untreated control and 2% DMSO-treated hPSCs. Error bars: SEM of 2–4 biological replicates. Scale bar: 50 µm. *, $p \leq .05$; **, $p \leq .01$ under one-way analysis of variance followed by a Tukey's post hoc test for multiple comparisons.

analyses (Supporting Information Fig. S5). Expression patterns for genes commonly implicated in cell division or regulating early differentiation of hPSCs [6, 7] are shown for DMSO-treated hPSCs compared with untreated control hPSCs as cells progress through the cell cycle (Supporting Information Fig. S5). Human embryonic and pluripotent stem cells are known to have minimal regulatory control across phases of the cell cycle and be refractory toward growth inhibitory signals. As a result, oscillation of gene expression across phases of the cell cycle is modest in hPSCs [25–27]. However, activation of checkpoint controls has been shown to be associated with improved cell cycle regulation and differentiation potential. Consistent with

this, we observed a correlation between DMSO treatment and increased cell cycle phase oscillation across all genes. Mean SD across all genes between early G1 and late G1 was 2.05 TPM in control hPSCs and 3.72 TPM for DMSO-treated hPSCs. Although the transition between late G1 and SG2M was relatively consistent across the two groups, mean SD across all genes between SG2M and early G1 was 1.34 TPM in control hPSCs and 2.68 TPM for DMSO-treated hPSCs. Interestingly, pluripotency genes (GO Term GO:0019827 Pluripotency Genes; FDR = 8.50e−1 by Fischer's exact test) were not altered, suggesting that the DMSO effect on improved differentiation is not mediated by altering the expression of the pluripotency network (Supporting Information Fig. S6A–S6C). A transient 24 hours DMSO treatment also does not affect cell toxicity as cell viability is comparable in untreated control and DMSO-treated hPSCs prior to differentiation (Supporting Information Fig. S6D, S6E), consistent with prior reports [2, 15].

Given the convergence toward PI3K, we next investigated whether inhibiting PI3K would mimic the DMSO treatment and increase the multilineage differentiation potential of hPSCs. To suppress PI3K signaling, we treated H9 hPSCs with small molecule PI3 kinase inhibitors (LY294002 and Wortmannin) for 24 hours and subsequently induced differentiation into the ectodermal, mesodermal, and endodermal germ layers using previously published protocols (Fig. 3A). Following directed differentiation, protein expression of germ layer specific genes [28], Sox1 (ectoderm), Brachyury (mesoderm), and Sox17 (endoderm) were assessed by immunostaining. Treatment with the PI3K inhibitors increased subsequent differentiation capacity across all germ layers in a dose-dependent manner (Fig. 3B, 3C) and enhanced expression of several lineage-specific genes (Fig. 3D). Similar improvements in differentiation were observed in another hPSC line, HUES6, known to have a very poor propensity for differentiation [4] (Supporting Information Fig. S7). Together, these results show that understanding gene trajectories in the cell cycle of hPSCs can highlight important signaling mechanisms regulating hPSC differentiation.

Discussion

Strikingly, although DMSO is an agent with pleiotropic effects [29, 30], here, we show that a short 24 hours treatment of hPSCs targets 2,972 genes in an orchestrated manner, particularly those controlling cell division and early developmental pathways. Genes are periodically expressed because there is special need for the gene products at particular points in the cell cycle [31]. Genes associated with cytoskeletal, cilium assembly, and cell adhesion factors were especially subject to regulation by the DMSO treatment in the SG2M phases, characteristic of a time when cells may need to duplicate centrioles in the S phase, change shape during mitosis, or exit the mitotic cycle to differentiate. Many of the targeted pathways play critical roles during embryogenesis including Wnt, bone morphogenic factor, nodal growth differentiation factor, fibroblast growth factor, Hippo, epidermal growth factor, vascular endothelial growth factor, and platelet derived growth factor as well as the downstream signaling pathways such as MAPK, Trk receptor, and PI3K [32]. Integration of these signaling pathways coordinates a number of developmental processes, including proliferation, fate determination, differentiation, apoptosis, migration, adhesion, and cell shape, to ultimately affect organogenesis. Most of the pathways that were regulated by DMSO converged on PI3 kinase signaling. Concordantly, suppression of PI3K signaling increased differentiation propensity across all germ layers in hPSCs, highlighting the utility of the genome-wide profiling approach used here to dissect out important signaling mechanisms regulating the developmental potential of pluripotent stem cells. This work is consistent with prior studies showing that PI3K-dependent signals promote embryonic stem cell proliferation and supports the notion that each phase of the cell cycle is important in performing distinct roles to orchestrate stem cell fate [26, 33]. Many of the signaling pathways and effects on metabolic function and cell adhesion identified here were also reported to play important regulatory roles during early transitions in pig embryonic development in recent work [34], suggesting shared mechanisms across species.

It would be interesting to investigate if improvements in terminal differentiation or enhancements in clustered regularly interspaced short palindromic repeats-mediated genome editing of hPSCs following a 24 hours DMSO treatment [35] may be due to changes in the molecular properties elicited on the pluripotent cell cycle.

Conclusion

Our data yield novel insights on the transcriptional and signaling dynamics during early transitory states in hPSCs that could be a useful point of focus in studying embryonic development. Targeting these early modes of regulation may put hPSCs on a better trajectory for differentiation and ultimately improve their utility for regenerative medicine.

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Author Contributions

A.S.: collection and/or assembly of data, all RNA-sequencing and bioinformatics data analyses and interpretation, experimental design, manuscript writing; J.L.: collection and/or assembly of data, experimental design, data analysis and interpretation of experimental validation studies; C.N.: collection and/or assembly of data; W.G.: experimental design; A.K.: data analysis and interpretation, experimental design, final approval of manuscript; S.C.: conception and experimental design, data analysis and interpretation, financial support, provision of study material, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

W.G. declared Inventor or patent holder with 10× genomics, consultant/advisory role and stock ownership with Guardant Health. The other authors indicated no potential conflicts of interest.

Data Availability Statement

Datasets associated with this article can be downloaded from GEO: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126788.

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