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Brief Report

Reprogramming of Human Primary Somatic Cells by OCT4 and Chemical Compounds

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Induced pluripotent stem cell (iPSC) technology, i.e. reprogramming somatic cells into pluripotent cells that closely resemble embryonic stem cells (ESCs) by introduction of defined transcription factors (TFs), holds great potential in biomedical research and regenerative medicine (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Various strategies have been developed to generate iPSCs with fewer or no exogenous genetic manipulations, which represent a major hurdle for iPSC applications (Yamanaka, 2009). With the ultimate goal of generating iPSCs with a defined small molecule cocktail alone, substantial effort and progress have been made in identifying chemical compounds that can functionally replace exogenous reprogramming TFs and/or enhance the efficiency and kinetics of reprogramming (Shi et al., 2008; Huangfu et al., 2008; Lyssiotis et al., 2009; Ichida et al., 2009; Maherali and Hochedlinger, 2009; Lin et al., 2009; Li et al., 2009; Esteban et al., 2010). To date, only neural stem cells (NSCs), which endogenously express SOX2 and cMYC at a high level, have been reprogrammed to iPSCs by exogenous expression of just OCT4 (Kim et al., 2009). However, human fetal NSCs are rare and difficult to obtain. It is therefore important to develop reprogramming conditions for other more accessible somatic cells. Here we report a small molecule cocktail that enables reprogramming of human primary somatic cells to iPSCs with exogenous expression of only OCT4. In addition, mechanistic studies revealed that modulation of cell metabolism from mitochondrial oxidation to glycolysis plays an important role in reprogramming.

Among several readily available primary human somatic cell types, keratinocytes can be isolated easily from human skin or hair follicle, and therefore represent an attractive cell source for reprogramming. Keratinocytes also endogenously express *KLF4* and *cMYC*, and can be reprogrammed efficiently using the conventional four TFs or three TFs (without *cMYC*) (Aasen et al., 2008; Maherali et al., 2008). More recently, we reported that dual inhibition of TGFβ and MAPK/ERK pathways using small molecules (SB431542 and PD0325901, respectively) provided significantly enhanced conditions for reprogramming of human fibroblasts with the four TFs (i.e., OSKM) (Lin et al., 2009). We have also shown that this dual pathway inhibition can also enhance reprogramming of human keratinocytes by two exogenous TFs (i.e., OK) with two small molecules, Parnate (an inhibitor of lysine-specific demethylase 1) and CHIR99021 (a GSK3 inhibitor) (Li et al., 2009). With a goal of OCT4-only reprogramming, we developed a stepwise strategy for refining reprogramming conditions and identifying additional small molecules that enhance reprogramming.

We first attempted to further optimize the reprogramming process using four or three TFs (i.e., OSKM or OSK) in neonatal human epidermal keratinocytes (NHEKs) by testing various inhibitors of TGFβ and MAPK pathways at different concentrations using previously reported human iPSC characterization methods (Lin et al., 2009). Encouragingly, we found that the combination of 0.5 μM A-83-01 (a more potent and selective TGFβ receptor inhibitor) and 0.5 μM PD0325901 was more effective than previous small molecule combinations at enhancing reprogramming of human keratinocytes transduced with OSKM or OSK (Figure 1A). Remarkably, when we reduced the viral transduction to only two factors (OK), we could still generate iPSCs from NHEKs when they were treated with 0.5 μM A-83-01 and 0.5 μM PD0325901, although with low efficiency. We then began screening additional small molecules from a collection of known bioactive compounds at various concentrations as previously reported (Shi et al., 2008). Among more than 50 compounds tested, we found that a small molecule activator of 3′-phosphoinositide-dependent kinase-1 (PDK1), PS48 (5 μM), which has not previously been reported to have reprogramming activity, can enhance reprogramming efficiency by about 15-fold. Interestingly, we also found that 0.25 mM sodium butyrate (NaB, a histone deacetylase inhibitor) is much more reliable and efficient than the previously reported 0.5 mM VPA for the generation of iPSCs under OK conditions (Figure 1B). Subsequent follow-up studies demonstrated that a combination of 5 μM PS48 and 0.25 mM NaB could further enhance the reprogramming efficiency over 25-fold (Figure 1B).

With such high efficiency of reprogramming NHEKs with two TFs, we explored the possibility of generating iPSCs with OCT4 alone by refining combinations of these small molecules during different treatment windows. Primary NHEKs were transduced with OCT4 and treated with the chemicals. We found that small TRA-1-81-positive iPSC colonies resembling hESCs (four to six colonies out of 1,000,000 seeded cells) appeared in OCT4-infected NHEKs that were treated with 0.25 mM NaB, 5 μM PS48, and 0.5 μM A-83-01 during the first 4 weeks, followed by treatment with 0.25 mM NaB, 5 μM PS48, 0.5 μM A-83-01, and 0.5 μM PD0325901 for another 4 weeks. These TRA-1-81-positive iPSC colonies grew larger in conventional hESC culture media and could be serially passaged to yield stable iPSC clones that were characterized further. We also found that

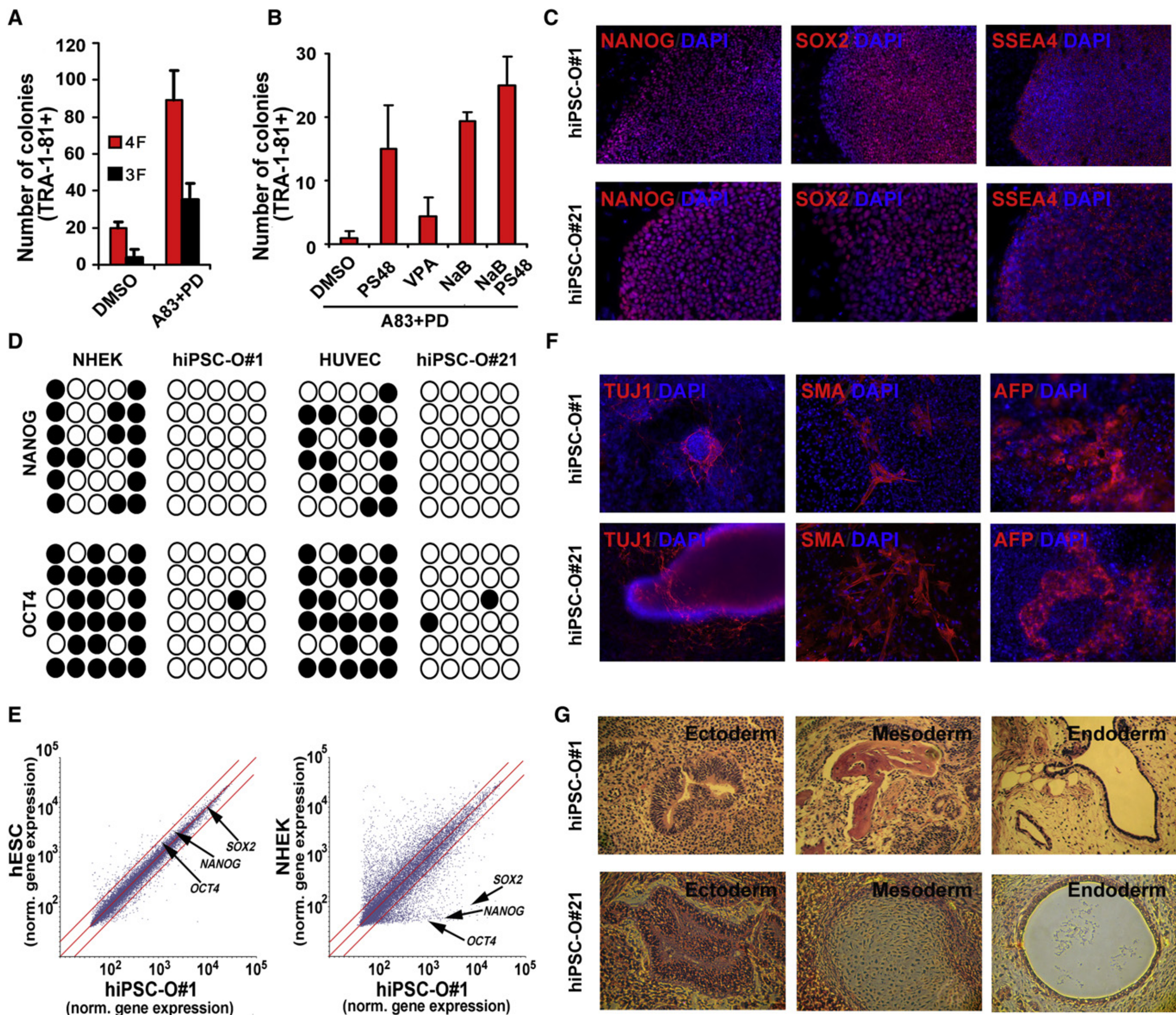

Figure 1. Generation and Characterizations of Human-Induced Pluripotent Stem Cells from Primary Somatic Cells by a Single Gene, OCT4, and Small Molecules

(A) Treatment with 0.5 μM A-83-01 (A83) and 0.5 μM PD0325901 (PD) significantly improved generation of iPSCs from primary human keratinocytes transduced with either four TFs (4F, OKSM) or three TFs (3F, OKS). NHEKs were seeded at a density of 100,000 transduced cells per 10 cm dish.

(B) Further chemical screens identified PS48, NaB, and their combination as compounds that can substantially enhance reprogramming of primary human keratinocytes transduced with two TFs (OK). NHEKs were seeded at a density of 100,000 transduced cells per 10 cm dish.

(C) The established human iPSC-O cells by OCT4 and small molecules from NHEKs and HUVECs express typical pluripotency markers, including NANOG (red), SOX2 (red), and SSEA4 (red). Nuclei were stained with DAPI (blue).

(D) Methylation analysis of the *OCT4* and *NANOG* promoters by bisulfate genomic sequencing. Open circles and closed circles indicate unmethylated and methylated CpGs, respectively, in the promoter regions.

(E) Scatter plots comparing global gene expression patterns between hiPSC-O cells and NHEKs, and hESCs. The positions of the pluripotency genes *OCT4*, *NANOG*, and *SOX2* are shown by arrows. Red lines indicate the linear equivalent and 2-fold changes in gene expression levels between the samples.

(F) Human iPSC-O cells could effectively differentiate in vitro into cells in the three germ layers, including neural ectodermal cells (βIII tubulin^{+}), mesodermal cells (SMA^{+}), and endodermal cells (AFP^{+}), using the EB method.

(G) Human iPSC-O cells could effectively produce full teratoma, which contained differentiated cells in the three germ layers, in *SCID* mice. See also Figures S1 and S2, Tables S1 and S2, and Movie S1.

OCT4-only iPSCs could be generated from adult human epidermal keratinocytes (AHEKs) by addition of 2 μM Parnate and 3 μM CHIR99021 (which had been shown to improve reprogramming of NHEKs under OK condition) to this chemical cocktail (Table S1 available online).

After this successful reprogramming of primary human keratinocytes to iPSCs by OCT4 and small molecules, we applied the same conditions to other human primary cell types, including human umbilical vein endothelial cells (HUVECs) and amniotic fluid-derived cells (AFDCs). Likewise, TRA-1-81-positive iPSC colonies

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appeared in OCT4-infected HUVECs and AFDCs that were treated with chemicals for 5–6 weeks. Remarkably, reprogramming of HUVECs and AFDCs was more efficient and faster than reprogramming of NHEKs using the OCT4 and small molecules protocol (Table S1). Finally, two clones of iPSC-O cells from each cell type were expanded for over 20 passages under conventional hESC culture condition and characterized further (Table S2).

The stably expanded hiPSC-O cells were morphologically indistinguishable from hESCs, stained positive for alkaline phosphatase (ALP), and expressed typical pluripotency markers, including OCT4, SOX2, NANOG, TRA-1-81, and SSEA4, as detected by immunocytochemistry (ICC) (Figures 1C, S1A, S1C, and data not shown). In addition, RT-PCR analysis confirmed the expression of the endogenous human *OCT4*, *SOX2*, *NANOG*, *REX1*, *UTF1*, *TDGF2*, and *FGF4* genes, and the silencing of exogenous *OCT4* (Figure S2A available online). Furthermore, bisulfite sequencing analysis revealed that the *OCT4* and *NANOG* promoters of hiPSC-O cells are largely demethylated, providing further evidence for reactivation of the pluripotency transcription program in these cells (Figure 1D). Global gene expression analysis of hiPSC-O cells, NHEKs, and hESCs showed that hiPSC-O cells are distinct from NHEKs (Pearson correlation value: 0.87) and most similar to hESCs (Pearson correlation value: 0.98) (Figure 1E). Genotyping analysis showed that hiPSC-O cells only contained the *OCT4* transgene without the contamination of transgenes *KLF4* or *SOX2* (Figure S2B). Karyotyping results demonstrated that hiPSC-O maintained a normal karyotype during the whole reprogramming and expansion process (Figure S2C), and DNA fingerprinting tests excluded the possibility that they arose from hESC contamination in the laboratory (Figure S2D).

To examine the developmental potential of the hiPSC-O cells, they were differentiated in vitro using a standard embryoid body (EB) differentiation approach. ICC analyses demonstrated that the hiPSC-O cells could effectively differentiate into characteristic βIII-tubulin$^+$ neuronal cells (ectoderm), SMA$^+$ mesodermal cells, and AFP$^+$ endodermal cells (Figures 1F, S1B, and S1D). Quantitative PCR analyses further confirmed the expression of these and additional lineage-specific marker genes characteristic of ectodermal (*βIII-tubulin* and *NESTIN*), mesodermal (*MSX1* and *MLC2a*), and endodermal (*FOXA2* and *AFP*) cells (data not shown). After the EB protocol, the hiPSC-O cells could also give rise to rhythmically beating cardiomyocytes (Movie S1 available online). To test the in vivo pluripotency of the cells, they were transplanted into *SCID* mice. After 4–6 weeks, the hiPSC-O cells effectively generated typical teratomas containing derivatives of all three germ layers (Figure 1G). Collectively, these in vitro and in vivo characterizations demonstrated that a single transcription factor, OCT4, combined with a defined small molecule cocktail is sufficient to reprogram several human primary somatic cell types to iPSCs that are morphologically, molecularly, and functionally similar to pluripotent hESCs.

We then investigated the molecular mechanism of action of the PDK1 activator PS48 in enhancing reprogramming. It has been shown that differentiated somatic cells typically use mitochondrial oxidation while pluripotent cells mainly use glycolysis for cell proliferation (Manning and Cantley, 2007; Kondoh et al., 2007; Vander Heiden et al., 2009; Prigione et al., 2010). Therefore, generation of iPSCs would appear to involve metabolic reprogramming from mitochondrial oxidation to glycolysis. Differential use of glycolytic metabolism over mitochondrial oxidation by pluripotent cells would favor pluripotency by promoting proliferation/cell cycle transitions with less oxidative stress. In addition, in highly proliferating cells oxidative phosphorylation would not be able to meet the demand of providing macromolecular precursors for cell replication, and would also generate a significant amount of reactive oxygen species that could induce excessive oxidative damages. On the other hand, glycolytic metabolism could more effectively generate macromolecular precursors, such as glycolytic intermediates for nonessential amino acids and acetyl-CoA for fatty acids, while providing sufficient energy to meet the needs of proliferating cells (Kondoh et al., 2007; Vander Heiden et al., 2009). In addition, growth factor signaling pathways-AKT activation, hypoxic conditions/HIF-1α, and the reprogramming factor MYC all regulate various aspects of cellular metabolism, including the upregulation of glucose transporters and metabolic enzymes of glycolysis, such as GLUT1, HK2, and PFK1 (Gordan et al., 2007; DeBerardinis et al., 2008). Interestingly, a hypoxic environment and its effector, HIF-1α activation, both of which have been closely linked to promoting glycolytic metabolism, were reported to improve reprogramming efficiency for both mouse and human cells (Yoshida et al., 2009). Moreover, MYC expression/activity was also shown to play an essential role in promoting glycolytic metabolism and promoting reprogramming efficiency (Vander Heiden et al., 2009). Together, these studies suggested that one potential mechanism of MYC, hypoxic condition/HIF-1α, and growth factor/AKT pathway activation in enhancing reprogramming could involve converging on an essential role in regulating glycolytic metabolism.

Because PS48 is an allosteric small molecule activator of PDK1 that can lead to downstream AKT activation (Hindie et al., 2009), we hypothesized that PS48 may facilitate a metabolic conversion from mitochondrial oxidation to glycolysis during the reprogramming process, as discussed above. Supporting this notion, we found that treatment with PS48 activated downstream AKT/PKB (Figure 2A), upregulated expression of several key glycolytic genes (Figure 2D), and consequently enhanced glycolysis as measured by increased lactate production (Figure 2E). The effects of PS48 on promoting reprogramming and conversion to glycolysis could be blocked by either a specific PDK1 inhibitor (UCN-01) or a specific glycolysis inhibitor (2-Deoxy-D-glucose, or 2-DG) (Figures 2B and 2E). Consistently, we also found that UCN-01 inhibited glycolytic gene expression in NHEKs (Figure 2D), and either UCN-01 or 2-DG on their own blocked the reprogramming process (Figure 2C). Importantly, and consistent with the idea that somatic cells differentially use mitochondrial oxidation for cell proliferation, inhibition of glycolysis by 2-DG did not affect somatic cell proliferation (Figure 2F). This result ruled out the possibility that the glycolysis inhibitor's effect on reprogramming results from an effect on cell growth/viability. In addition, a time course study on glycolysis

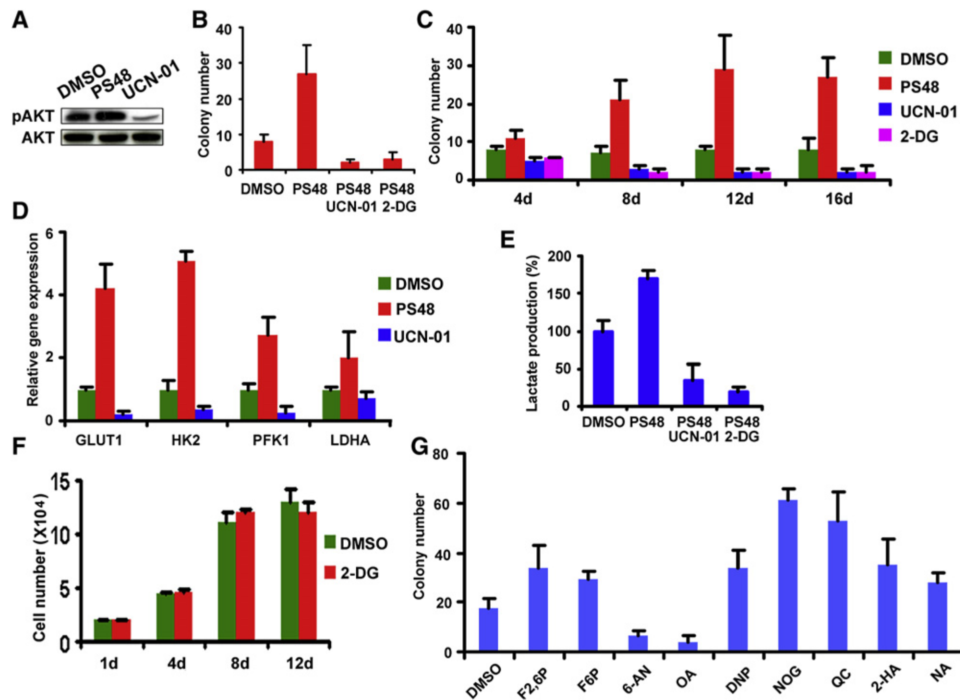

Figure 2. A Metabolic Switch toward Glycolysis Facilitates Reprogramming

(A) PS48 treatment activated PDK1 activity. The phosphorylation of AKT (Thr-308) after PS48 (5 μM) or UCN-01(20 nM) treatment was analyzed by western blotting.

(B) PS48 enhanced reprogramming of NHEKs, while UCN-01 (20 nM) or 2-Deoxy-D-glucose (2-DG) (10 mM) inhibited PS48's reprogramming enhancing effect. OSK-transduced NHEKs were seeded at a density of 100,000 cells per well and treated with compounds for 4 weeks, and then TRA-1-81-positive colonies were counted.

(C) The time course study of chemical treatment on reprogramming. OSK-transduced NHEKs were seeded at a density of 100,000 cells per well and treated with compounds for different times, and then TRA-1-81-positive colonies were counted at the end of 4 weeks after transduction.

(D) Chemical treatment affected the expression of several key glycolytic genes, including *GLUT1*, *HK2*, *PFK1*, and *LDHA*.

(E) PS48 treatment facilitated/activated a metabolic switch to glycolysis, while its effect could be blocked by UCN-01 or 2-DG. NHEKs were treated with compounds for 8 days and then lactate production in the medium was measured as a typical index of glycolysis.

(F) Inhibition of glycolysis by 2-DG did not affect the cell proliferation of somatic cells. NHEKs were treated with DMSO (control) or 2-DG, and cell number was counted at the indicated time points.

(G) Known small molecules that have been widely used to modulate mitochondrial oxidation, glycolysis metabolism, or HIF activation also showed corresponding consistent effects on reprogramming. OSKM-transduced HUVECs were seeded at a density of 20,000 cells per well and treated with the metabolism modulating compounds for 3 weeks, and TRA-1-81-positive colonies were counted. F2,6P, 10 mM Fructose 2,6-bisphosphate; F6P, 10 mM Fructose 6-phosphate; 6-AN, 10 μM 6-aminonicotinamide; OA, 10 μM oxalate; DNP, 1 μM 2,4-dinitrophenol; NOG, 1 μM N-oxaloylglycine; QC, 1 μM Quercetin; 2-HA, 10 μM 2-Hydroxyglutaric acid; NA, 10 μM nicotinic acid. DMSO was used as a control.

modulators' effect on reprogramming revealed that only 8 day treatment with these glycolysis modulators was sufficient to affect reprogramming efficiency (Figure 2C). This finding suggests that early steps in the reprogramming process were affected by these specific glycolysis modulators because there were no iPSCs generated at this time point (8 days). Moreover, several known small molecules that have been widely used to modulate mitochondrial oxidation (2,4-dinitrophenol), glycolytic metabolism (Fructose 2,6-bisphosphate and oxalate), or more specifically HIF pathway activation (N-oxaloylglycine and Quercetin) also showed corresponding effects on reprogramming: i.e., compounds that promote glycolytic metabolism enhance reprogramming (such as 2,4-dinitrophenol and N-oxaloylglycine), whereas compounds that block glycolytic metabolism inhibit reprogramming (such as oxalate) (Figure 2G) (Hewitson and Schofield, 2004; Pelicano et al., 2006). In conclusion, these results collectively indicated that a metabolic switch to anaerobic glycolysis is an important step in reprogramming somatic cells to pluripotent stem cells.

These studies have a number of important implications: (1) although human fetal NSCs were previously reprogrammed to iPSCs by ectopic expression of OCT4 alone, our study now shows that iPSCs can be derived from readily available primary human somatic cells (e.g., keratinocytes) transduced with a single exogenous reprogramming gene, OCT4. (2) The identification of small molecule cocktail that we used, which functionally replaces three master TFs all together (i.e., SOX2, KLF4, and MYC) in enabling the generation of iPSCs with OCT4 alone, represents an additional step toward the overall goal of reprogramming with only small molecules. (3) One way in which PS48 enhances reprogramming appears to be facilitating the metabolic conversion from mitochondrial oxidation, which is mainly used by adult somatic cells, to glycolysis, which is mainly used by pluripotent cells. Modulation of cellular metabolism by small molecules to either enhance reprogramming or inhibit pluripotent cell proliferation may well have additional applications in the future development of iPSC technology.

ACCESSION NUMBERS

The GEO accession number is GSE25218 for our microarray data in this manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures, two tables, Supplemental Experimental Procedures, and lists of primers and antibodies used and can be found with this article online at doi:10.1016/j.stem.2010.11.015.

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