Inhibition of Transforming Growth Factor β (TGF-β) Signaling can Substitute for Oct4 Protein in Reprogramming and Maintain Pluripotency*

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Background: Pluripotent stem cells (PSCs) have been used widely to study molecular mechanisms involved in early embryonic development. The signaling pathways orchestrated by leukemia inhibitor factor/STAT3, Wnt/β-catenin, and FGF/MEK/ERK play key roles in the generation of pluripotency. However, the function of TGF-β signaling in this process remains elusive. Here we show that inhibiting TGF-β signaling with its inhibitor SB431542 can substitute for Oct4 during reprogramming. Moreover, inhibiting TGF-β signaling can sustain the pluripotency of iPSCs and ES cells through modulating FGF/MEK/ERK signaling. Therefore, this study reveals a novel function of TGF-β signaling inhibition in the generation and maintenance of PSCs.

Results: The TGF-β inhibitor SB431542 substitutes for Oct4 in reprogramming and maintains pluripotency. LIF and two inhibitors, PD0325901 (PD03) and CHIR99021 (CHIR), maintain ES cells through the activation of Stat3 (3). PD03 supports the self-renewal of ES cells by inhibiting MEK (4). CHIR inhibits glycogen synthase kinase 3 (Gsk3), leading to the activation of canonical Wnt/β-catenin signaling (5). Therefore, the LIF/STAT3, FGF/MEK/ERK, and Wnt/β-catenin pathways are involved in the maintenance of pluripotency. However, whether other signaling pathways are involved in the maintenance of pluripotency of ES cells is unknown.

Conclusion: TGF-β signaling inhibition promotes generation and maintenance of PSCs.

Significance: Our study reveals a novel function of TGF-β signaling in the generation of PSCs.

Mouse pluripotent stem cells (PSCs), such as ES cells and induced PSCs (iPSCs), are an excellent system to investigate the molecular and cellular mechanisms involved in early embryonic development. The signaling pathways orchestrated by leukemia inhibitor factor (LIF), Wnt/β-catenin, and FGF/MEK/ERK play key roles in the generation of pluripotency. However, the function of TGF-β signaling in this process remains elusive. Inhibiting TGF-β signaling with its inhibitor SB431542 can substitute for Oct4 during reprogramming. Moreover, inhibiting TGF-β signaling can sustain the pluripotency of iPSCs and ES cells through modulating FGF/MEK/ERK signaling. Therefore, this study reveals a novel function of TGF-β signaling inhibition in the generation and maintenance of PSCs.

Embryonic development is a highly complicated event and is under strict regulations. However, how the development of early embryos occurs normally remains largely unclear. Pluripotent stem cells (PSCs), such as ES cells, which retain the ability to generate almost all types of cells, have been used widely to study the molecular mechanisms involved in early embryonic development. Mouse ES cells were initially derived from embryonic day 3.5 blastocysts and cultured in serum-containing medium with leukemia inhibitor factor (LIF) (1). Subsequently, Smith and co-workers (2) demonstrated that ES cells can be derived and sustained under serum-free conditions with LIF and two inhibitors, PD0325901 (PD03) and CHIR99021 (CHIR). LIF maintains ES cells through the activation of Stat3 (3). PD03 supports the self-renewal of ES cells by inhibiting MEK (4). CHIR inhibits glycogen synthase kinase 3 (Gsk3), leading to the activation of canonical Wnt/β-catenin signaling (5). Therefore, the LIF/STAT3, FGF/MEK/ERK, and Wnt/β-catenin pathways are involved in the maintenance of pluripotency. However, whether other signaling pathways are involved in the maintenance of pluripotency of ES cells is unknown.

In addition to being obtained from early embryos, pluripotent stem cells can also be generated from differentiated cells. Transducing Oct4, Sox2, Klf4, and c-Myc into somatic cells can lead to induced pluripotent stem cells (iPSCs) (6, 7). The iPSCs can be generated under serum conditions or serum-free conditions with PD03 and CHIR (8). Under serum conditions, multiple signaling pathways participate in reprogramming. For example, the activation of Wnt or FGF signaling can promote the reprogramming of somatic cells to pluripotency (9, 10). Moreover, the bone morphogenetic proteins (BMPs) in serum have been identified as the key signaling factors in interrupting reprogramming (11). Notably, somatic cells transduced with the reprogramming factors often fail to be fully reprogrammed, but these intermediate cells can be reprogrammed to complete iPSCs in serum-free medium (8). Under this condition, FGF/MEK/ERK signaling inhibition by PD03 induces the transition to complete pluripotency, but Wnt signaling activation by CHIR alone has no discernible effect (8). However, whether other signaling pathways are involved in the generation of iPSCs remains unknown.

The TGF-β signaling pathway affects a variety of processes, including cell proliferation, differentiation, and development (13–15). However, the role of this pathway in maintaining the
pluripotency of mouse ES cells remains controversial (16–23). The function of TGF-β signaling in reprogramming has been largely elucidated. Under serum conditions, the inhibition of this pathway improves the efficiency of generating iPSCs (24, 25). Moreover, the addition of TGF-β signaling inhibitors in serum-containing medium can replace Sox2 and c-Myc in reprogramming, but they cannot substitute for Oct4 or Klf4 (24, 25). Nevertheless, under serum-free conditions, whether this signaling pathway participates in reprogramming is unclear.

In this study, we showed that the inhibition of TGF-β signaling by SB431542 (SB43), a small molecule, can substitute for Oct4 to reprogram mouse embryonic fibroblasts (MEFs) into iPSCs. Moreover, the repression of TGF-β signaling can sustain the pluripotency of iPSCs and ES cells. Mechanistic studies show that TGF-β signaling inhibition can modulate the FGF/MEK/ERK pathway through reducing ERK phosphorylation in ES cells.

**EXPERIMENTAL PROCEDURES**

*Cell Culture—* MEFs were derived from embryonic day 13.5 C57BL/6 mouse embryos. Limbs and tail tips were cut into pieces and trypsinized. Isolated MEFs were maintained in DMEM containing 10% FBS, expanded until passage 2, and then used for reprogramming.

The mouse ES cell lines R1 and E14Tg2a and iPSC cells were used in this study. ES cells and iPSCs were cultured under feeder-free condition with LIF in either serum-containing medium with knockout DMEM containing 10% FBS or serum-free N2B27 medium conditions supplemented with CHIR99021 (3 μM, Calbiochem), PD0325901 (1 μM, Tocris), or SB431542 (5 μM, Tocris). N2B27 medium consisted of DMEM/F12 (Invitrogen) and Neurobasal (Invitrogen) at a 1:1 ratio, 1% N2 supplement (Invitrogen), 2% B27 (Invitrogen), 2 mM GlutaMAX (Invitrogen), and 0.055 mM β-mercaptoethanol (Invitrogen).

*iPSCs Generation—* For iPSCs generation, lentiviral packaging and transfection were performed as described previously (27). In brief, 293T cells were transfected with FUW-tet-on-hOCT4, hSOX2, hKLF4, hMYC (OSKM), and rtTA plasmids (Addgene) separately with the packaging plasmids VSVG and hOCT4, hSOX2, hKLF4, hc-MYC (OSKM), and rtTA plasmids (27). In brief, 293T cells were transfected with FUW-tet-on-ingen and transfection were performed as described previously. After 12 h, medium was switched to N2B27 to induce differentiation. After 5–7 days, cells were assayed for expression of lineage-specific genes.

For teratoma formation, 7–9 × 10⁶ iPSCs were injected into nude mice. Paraffin sections of teratomas were performed about 5 weeks after injection and stained with H&E.

*In vitro differentiation of ES cells grown under different culture conditions was performed by embryoid body formation. ES cell serum-free neural differentiation (5% knockout serum replacement medium) and unbiased differentiation in serum-containing medium (10% FBS) were performed as described previously (29–31). Briefly, ES cells were dissociated into single cells after treatment with 0.05% trypsin-EDTA at 37 °C for 2 min. Single cells were aggregated in Petri dishes at a density of 1 × 10⁵ cells/ml in differentiation medium. Differentiation day 0 indicates the day on which the ES cells were seeded to differentiate. Embryoid bodies were collected every other day and analyzed by quantitative reverse-transcription PCR (qRT-PCR).

**AP Staining and Immunofluorescence Staining—** AP staining was performed as described previously (30). Briefly, cells were washed with PBS three times and stained with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Promega) for 10 min.

For immunofluorescence staining, samples were washed once with PBS and fixed with a 4% pure formaldehyde solution (Sigma-Aldrich) in PBS for 20 min, followed by two washes with PBS. Blocking and permeabilization were done with a 3% BSA and 0.3% Triton X-100 (Sigma-Aldrich) solution in PBS for 1 h at room temperature. All primary antibodies were diluted in 0.3% Triton X-100 and incubated overnight at 4 °C. After 1 h of washing with PBS, samples were incubated with Alexa Fluor 488-, Alexa Fluor 555-, or Alexa Fluor 647-conjugated secondary antibodies (Invitrogen) for 2 h at room temperature. The following primary antibodies were used. Mouse monoclonal antibodies included anti-Oct4 (1:200, Santa Cruz Biotechnology), anti-Cytokeratin18 (CK18) (1:200, Abcam), anti-SSEA-1 (1:200, Santa Cruz Biotechnology), and anti-Tuj1 (1:500, Covance). The rabbit polyclonal antibody was anti-Nanog (1:400, Cell Signaling Technology). The goat polyclonal antibodies were anti-T (1:200, Santa Cruz Biotechnology) and anti-Gata6 (10 μg/ml, R&D Systems).

**Western Blotting—** Western blotting was performed as described previously (32, 33). Briefly, cells were lysed in cell lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% NaDOC, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA, 0.25 mM phenylmethanesulfonyl fluoride, and a mixture of protease inhibitors. Lysate samples were boiled for 10 min, separated by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) BSA in PBS at room temperature for 1 h and incubated with primary antibodies at room temperature for a further 1 h. The following primary antibodies were used. Anti-phosphorylated Smad (pSmad)1/5/8 (1:2000, Cell Signaling Technology), anti-Smad1 (1:2000, Cell Signaling Technology), anti-pERK1/2 (1:1000, Cell Signal-
Inhibition of TGF-β Signaling Promotes the Generation of iPSCs—To obtain iPSCs from differentiated cells under serum-free conditions, dox-inducible Oct4, Sox2, Klf4, and c-Myc (OSKM) were transduced into MEFs by lentiviral vectors. Three days after transduction, one group of cells was cultured in serum-free N2B27 medium containing PD03, CHIR, and VPA (VPA/PD03/CHIR, VPC) (34). The other group of cells were transduced with pSilencer shRNAs with the packaging plasmids VSVG and Δ8.9. Viruses were collected by ultracentrifugation 72 h after transfection. 2 × 10^5 MEFs were plated on a Matrigel-coated dish and infected with viral supernatants (multiplicity of infection = 4).

RNA Preparation and qRT-PCR Analysis—Total RNA was extracted from cultured cells using TRIzol (Invitrogen) reagent. One microgram of RNA was reverse-transcribed using Super-Script III reverse transcriptase (Invitrogen). qRT-PCR was performed with JumpStart Taq ReadyMix (Sigma-Aldrich). Reactions were carried out in triplicate using 1/40 concentration of the cDNA obtained as described above. The expression level of the transcript in each sample was normalized to Gapdh. The sequences of all primers used in this study are available upon request.

Statistics—All values are shown as mean ± S.D. The significance between groups was determined by Student’s t test.

RESULTS

Inhibition of TGF-β Signaling Promotes the Generation of iPSCs—To obtain iPSCs from differentiated cells under serum-free conditions, dox-inducible Oct4, Sox2, Klf4, and c-Myc (OSKM) were transduced into MEFs by lentiviral vectors. Three days after transduction, one group of cells was cultured in serum-free N2B27 medium containing PD03, CHIR, and VPA (VPA/PD03/CHIR, VPC) (34). The other group of cells was grown in serum-containing medium supplemented with VPA (serum/VPA) as the control. 24 days after induction, many AP-positive colonies were detected in the control serum/VPA medium, but AP-positive colonies were barely detectable under serum-free conditions. Interestingly, Oct4-positive colonies were observed in the control serum/VPA medium, whereas Nanog expression was detected in ~96% of the VSC-iPSCs but in less than 80% of the serum iPSCs (Fig. 1D). The homogeneous expression of Nanog, together with other characteristics, such as the expression of Oct4, Sox2, and AP, indicate that the VSC-iPSCs are high-quality iPSCs.

Somatic cells that are transduced with the reprogramming factors in serum-containing medium are often trapped in a pre-iPSC status and fail to fully reprogram (8). Nevertheless, these intermediate cells can be easily reprogrammed in serum-free medium containing an inhibitor of the MEK/ERK pathway (8). Moreover, either the early activation or the late inhibition of the FGF pathway can increase reprogramming efficiency in serum-containing medium (10). Therefore, a short-term treatment with serum and FGF2 before switching to the serum-free condition may improve the efficiency of generating iPSCs. To test this hypothesis, MEFs were first cultured in the initiation medium (pre-iPS medium containing serum, knockout serum replacer, and LIF) for 6 days with dox treatment. Thereafter, N2B27 medium containing different factors, such as VSC or VPC, was applied to induce the iPSCs, and serum-containing medium with dimethyl sulfoxide was used as the control (Fig. 1F). The VPA/SB43/CHIR/FGF2 (VSCF) treatment was same as the VSC treatment except for the addition of 10 ng/ml FGF2 in the first 6 days. Interestingly, the number of AP-positive iPS colonies generated under VSC conditions was significantly higher than under VPC conditions, and the number of AP-positive iPS colonies was the highest under VSCF conditions (Fig. 1, F and G). The quantitative data revealed that the reprogramming efficiency of the VSC treatment was ~40% of that in the serum-containing medium and was two times higher than that of the VPC treatment (Fig. 1G). Together, these results show that the inhibition of TGF-β signaling by SB43 can promote iPSC generation from mouse fibroblasts under serum-free conditions with or without pre-iPS medium treatment.

Inhibition of TGF-β Signaling Could Replace Oct4 during Reprogramming—Because VSCF treatment generated the most iPSC colonies (Fig. 1, F and G), we asked whether VSCF could substitute for any of the OSKM factors. After a short-term treatment with serum and FGF2, VSC was able to replace Sox2 or c-Myc, but it failed to induce iPSC colonies in the absence of Klf4 (data not shown), which is consistent with previous studies (24, 25). Surprisingly, VSCF was able to substitute for Oct4, and the reprogramming efficiency of VSCF was even higher than that of Oct4 (Fig. 2, A and D, left panel).

To explore which factor in VSCF was critical for Oct4 replacement, we examined the effect of withdrawing individual factors on the formation of AP-positive iPSC colonies in the presence of dox-induced Sox2, Klf4, c-Myc (SKM). Few colonies were observed in the absence of either VPA or SB43. In contrast, the removal of FGF2 or CHIR had little effect (Fig. 2, B and D, center panel). In addition, no colonies were detected...
with the transduced SKM alone, and a few colonies were observed under SKM conditions with either SB43 or VPA (Fig. 2, C and D, right panel). After treatment with SKM, SB43, and VPA (VS-SKM), the number of iPSC colonies was comparable with that of the OSKM-iPSCs (Fig. 2, A, C, and D). The colonies under SKM plus VS conditions could be observed 10 days after induction with dox, and the cells expressed Oct4 at day 24 (Fig. 2E). However, PD03 could not induce iPSC colonies in the presence of VPA (Fig. 2, C and D, right panel), suggesting that FGF/MEK/ERK signaling inhibition could not replace Oct4 during reprogramming.

Next, genomic DNA PCR assays confirmed that the iPSCs generated with VS-SKM were free of Oct4 transgene integration (Fig. 2F). VS-SKM iPSCs had a similar morphology to

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**FIGURE 1. Generation of iPSCs from MEFs.** MEFs were infected with lentiviruses encoding for dox-inducible OSKM. Relative reprogramming efficiencies were normalized to the efficiency observed under serum/VPA conditions. A, AP staining of reprogrammed MEFs under serum and serum-free conditions on day 24. B, the efficiency of iPSC colonies under different conditions. Three independent experiments were performed, and the results are presented as the mean ± S.D. *, p < 0.05; Student’s t test. V, VPA. C, AP staining and immunostaining of Oct4, Nanog, and SSEA1 of the iPSC cell colonies under VSC conditions. D, immunostaining of Oct4 and Nanog of the iPSCs generated under serum/VPA and VSC conditions. The iPSC colonies were picked and reseeded for one passage. The cells were cultured in LIF/serum or N2B27 medium containing LIF, SB43, and CHIR. E, the percentages of Nanog and Oct4 double-positive cells among Oct4 cells under different conditions. *, p < 0.05; Student’s t test. F, AP staining of reprogrammed MEFs after short-term serum supplementation. Different media were added sequentially as described under “Experimental Procedures.” G, the efficiency of iPSC cell colonies derived from MEFs. Three independent experiments were performed, and the results are presented as the mean ± S.D. *, p < 0.05; Student’s t test. Scale bars = 100 μm.
**TGF-β Signaling Inhibition Substitutes for Oct4 in Reprogramming**

**A**

|        | OSKM | SKM |
|--------|------|-----|
| DMSO   | DMSO | VSCF |

**B**

VSCF-1 Factor

|        | -VPA | -SB43 | -CHIR | -FGF2 |
|--------|------|-------|-------|-------|
| SKM    |      |       |       |       |

**C**

|        | DMSO | SB43 | VPA   |
|--------|------|------|-------|
| VPA+SB43 | VPA+PD03 |

**D**

Number of AP-positive cells

|          | Oct4 | DMSO | VSCF | VSCF | VSCF | VSCF | VS | V | S | V |
|----------|------|------|------|------|------|------|----|---|--|---|
| SKM      | *    |      |      |      |      |      |    |   |  |   |

**E**

Day 10 Day 24

**F**

OSKM IPSCs

| Oct4 | Sox2 | Klf4 | dMyc |
|------|------|------|------|
| line 1 |

VS-SKM IPSCs

| Oct4 | Sox2 | Klf4 | dMyc |
|------|------|------|------|
| line 2 |

| Oct4 | Sox2 | Klf4 | dMyc |
|------|------|------|------|
| line 3 |

**G**

OCT4/DAPI Nanog/DAPI SSEA1/DAPI

**H**

Neural epithelium Cartilage Adipocyte Gut-like epithelium

**I**

Ck18/DAPI T/DAPI Gata6/DAPI

**J**

Relative Oct4 expression

**K**

Relative mRNA expression

**L**

Relative mRNA expression

**M**

Relative Oct4 expression

**N**

VPA+ctrl shRNA VPA+Alk5 shRNAs

**O**

DMSO 616452 VPA+616452
mouse ES cells (data not shown). In addition, VS-SKM iPSCs were stable and remained positive for Oct4, Nanog, and SSEA1 after long-term passaging (Fig. 2G).

To evaluate the differentiation potential of the VS-SKM iPSCs, iPSCs were injected into nude mice, which were evaluated after about 5 weeks. iPSCs could give rise to teratomas that possessed various tissues originating from all three germ layers, including neural tissues, cartilage, adipocytes, and gut-like epithelium (Fig. 2H). Moreover, after monolayer differentiation in N2B27 medium for 5–7 days, cells positive for Ck18 (ectodermal marker), T (mesodermal marker), and Gata6 (endodermal marker) were detected (Fig. 2I). Therefore, these results show that VS-SKM iPSCs are pluripotent.

To investigate whether VPA and SB43 could activate the expression of endogenous Oct4 during reprogramming, Oct4 expression was monitored by qRT-PCR. Indeed, the expression of endogenous Oct4 was significantly activated 5–6 days post-induction (Fig. 2J). To determine the underlying mechanisms of how SB43 substituted for Oct4 in the presence of VPA, the expression of genes that have shown the ability to replace Oct4 was determined and compared with SkM MEFs, many genes were up-regulated in VS-SKM MEFs (Fig. 2K). In addition, the generation of iPSCs from mouse fibroblasts promotes mesenchymal-to-epithelial transition and suppresses epithelial-to-mesenchymal transition (40). In VS-SKM MEFs, several epithelial genes, such as Ocln, Epcam, and Cldn3, were up-regulated, and the mesenchymal genes Twist1, Snail, and Fn1 were down-regulated (Fig. 2L). These results suggest that SB43 might substitute for Oct4 by regulating the expression of multiple genes involved in mesenchymal-to-epithelial transition and activating the endogenous Oct4.

Because SB43 is a specific inhibitor of Alk5 (a TGF-β type I receptor) (35, 41), the inhibition of TGF-β signaling might substitute for Oct4 during reprogramming. To test this hypothesis, two different Alk5-specific shRNAs (shRNA-1 and shRNA-2) were introduced into mouse fibroblasts using a lentivirus, and both shRNAs could efficiently knock down the expression of Alk5 transcripts (Fig. 2M, left panel). Smad2 phosphorylation was decreased in fibroblasts transected with Alk5 shRNAs but not in those transfected with the control shRNA, indicating that the activity of TGF-β signaling was reduced with Alk5 shRNA treatment (Fig. 2M, right panel). Next we transduced fibroblasts with SkM and Alk5 shRNAs to assess the role of Alk5 during reprogramming. Approximately 30 AP-positive colonies were observed in 1.5 × 10⁵ cells treated with the Alk5 shRNAs. In contrast, no colonies were generated after treatment with the control shRNA (Fig. 2N). Consistently, when SkM-transduced fibroblasts were treated with 616452 (Rep-Sox), another Alk5 inhibitor, AP-positive colonies were readily detected with or without VPA (Fig. 2O). Collectively, these results demonstrate that inhibiting TGF-β signaling can substitute for Oct4 to reprogram mouse fibroblasts into iPSCs.

**The Inhibition of TGF-β Signaling Maintains the Pluripotency of ES Cells**—Because inhibiting the TGF-β pathway can substitute for Oct4 when generating iPSCs (Fig. 2), we asked whether TGF-β signaling participates in the maintenance of the pluripotency of PSCs. Therefore, PD03 was replaced by SB43 in serum-free medium plus CHIR and LIF, and the iPSC lines were cultured and passaged under these conditions (LIF/ SB43/CHIR, LSC) for more than 20 passages. All of these cells could produce colonies that were similar to those cultured in LIF/PD03/CHIR (LPC) medium, and AP-positive cells could be maintained without obvious morphological changes (Fig. 3A). Similar to LPC, the LSC medium could also maintain the pluripotency of two ES cell lines (R1 and E14tg2a, LSC-ES cells) for more than 20 passages (Fig. 3, B and C). The expression of pluripotency markers such as Oct4 and SSEA1 was sustained in LSC-ES cells (Fig. 3D). To determine the differentiation capability of these LSC-ES cells, the cells were dissociated and replated in N2B27 medium. After differentiation for ~7 days as a monolayer, cells expressing Tuj1 (an ectodermal marker), T (a mesodermal marker), and Gata6 (an endodermal marker) were readily detected (Fig. 3E). These results suggest that the inhibition of TGF-β signaling in the absence of the FGF/MEK/ERK pathway suppression could maintain pluripotency in mouse iPSCs and ES cells.

**Compared with ES cells cultured under LIF/PD03/CHIR conditions**, ES cells in serum-containing medium show less homogeneity in their expression of Nanog (36). To examine ESC homogeneity under LIF/SB43/CHIR conditions, E14tg2a ES cells were passaged several times under serum/LIF, LIF/ PD03/CHIR, or LIF/SB43/CHIR conditions, and immunostaining was used to detect the expression of Nanog and Oct4.
The expression of Oct4 was homogenous in ES cells under each condition. In contrast, the expression of Nanog was less uniform in ES cells cultured in serum-containing medium compared with cells grown under LIF/PD03/CHIR or LIF/SB43/CHIR conditions (Fig. 3, F and G).

The transcription profiles of ES cells cultured under different conditions revealed that the ES cells cultured under LIF/PD03/CHIR conditions exhibited lower expression levels of lineage-affiliated genes (42). Consistently, we also found that the expression of pluripotency genes such as Oct4, Nanog, Sox2, and Rex1 was high and that the expression of an epiblast marker, Fgf5, and lineage-specific marker genes was lower in LPC-ES cells and LSC-ES cells compared with ES cells in serum-containing medium (Fig. 3H).

FIGURE 3. PSCs can be maintained under LIF/SB43/CHIR conditions. A, morphology and AP staining of VS-SKM iPS cells under different culture conditions for more than 20 passages. B, morphology and AP staining of R1 ES cells grown under LIF/SB43/CHIR condition for more than 20 passages. C, morphology and AP staining of E14tg2a ES cells cultured under different conditions for more than 20 passages. D, immunostaining for Oct4 and SSEA1 of the E14tg2a cells under LSC conditions. E, three lineages of cells differentiated in vitro from the LSC E14tg2a cell line. Immunostaining detected cells expressing Tuj1 (an ectodermal marker), T (a mesodermal marker), and Gata6 (an endodermal marker). F, immunostaining for Oct4 and Nanog in the E14tg2a ES cell line after cultivation for over 10 passages under different conditions. G, the percentages of Nanog* Oct4+/Oct4−, *p < 0.05; Student’s t test. H, qRT-PCR of genes associated with pluripotency and the various germ layers. The E14tg2a ES cell line was cultivated for 5 passages in serum/LIF, LIF/SB43/CHIR, and LIF/PD03/CHIR. Two independent experiments were performed, and the results are presented as the mean ± S.D. Scale bars = 100 μm.
Next we asked whether the expression status would affect the differential kinetic of LSC-ES cells. To answer this question, ES cells that had been cultured in serum, LSC medium, or LPC medium were differentiated in an embryoid body system to examine their differentiation ability to form three germ layers. qRT-PCR was performed to profile the expression of marker genes during differentiation. Similar expression patterns were observed between LSC-ES cells and LPC-ES cells. Under both culture conditions, the expression of *Nanog* and *Rex1* was down-regulated when differentiation was initiated. Following the down-regulation of these pluripotency genes, the expression of *Fgf5* increased and then decreased (Fig. 4A). The expression levels of marker genes for the three germ layers, such as *Tbx6*, *Cxcr4*, and *Gata4* for mesoderm; *Sox17*, *Gata4*, and *Sox7* for endoderm; and *Pax6* and *Sox1* for ectoderm, were up-regulated gradually (Fig. 4, B–D). In contrast, the up-regulation of the mesoderm marker genes *Tbx6* and *Cxcr4* was obviously accelerated in ES cells that were cultured in serum-containing medium (Fig. 4B). These results suggest that LSC- and LPC-ES cells have similar differentiation potentials. Taken together, these data indicate that ES cells cultured in LSC and LPC are similar in some aspects.

**Inhibition of TGF-β Signaling Maintains Pluripotency by Reducing ERK Activity**—Because SB43 can replace PD03 and sustain the pluripotency of mouse ES cells in the presence of LIF and CHIR (Fig. 3), we asked how the inhibition of TGF-β signaling could substitute for the repression of the FGF/MEK/ERK pathway to support the pluripotency of ES cells cultured under serum-free conditions. Therefore, in the absence of CHIR, either the ERK inhibitor PD03 (1 μM), the MEK1 inhibitor PD18 (1 μM), or SB43 (1, 5, and 10 μM) was added to N2B27/LIF medium. SB43 (1 μM or 5 μM) could not effectively maintain the undifferentiated state of ES cells, which were characterized by the decreased expression of *Oct4* and *Nanog* as well as positive expression of AP. In contrast, similar to two inhibitors of FGF/MEK/ERK signaling, 10 μM SB43 could properly sustain ES cells (Fig. 5A). Interestingly, the mRNA expression levels of the pluripotency markers *Sox2*, *Rex1*, and *Klf4* were up-regulated by SB43 in a dose-dependent manner (Fig. 5B). Therefore, SB43 plus LIF can support the pluripotency of ES cells.

PD03 maintains the pluripotency of ES cells by inhibiting FGF/MEK/ERK signaling (2). Phosphorylated ERK (p-ERK) is an important effector that controls the transcriptional...
TGF-β Signaling Inhibition Substitutes for Oct4 in Reprogramming

A

|          | LIF                  | PD03 (1 μM) | PD18 (1 μM) | SB43 (1 μM) | SB43 (5 μM) | SB43 (10 μM) | DMSO | DMSO |
|----------|----------------------|-------------|-------------|-------------|-------------|--------------|------|------|
| Phase    |                      |             |             |             |             |              |      |      |
| Oct4/DAPI|                      |             |             |             |             |              |      |      |
| Nanog/DAPI|                     |             |             |             |             |              |      |      |
| AP       |                      |             |             |             |             |              |      |      |

B

- Sox2
- Klf4
- Rex1

C

- p-ERK1/2
- ERK1/2
- p-Smad2
- Smad2
- p-Smad1
- Smad1
- GAPDH

D

- Activin
- p-ERK1/2
- ERK1/2
- Tubulin

E

- Fra1
- Egr1
- Fos

F

- p-ERK1/2
- ERK1/2
- Serum+LIF
- LIF+CHIR+SB43
- LIF+CHIR-PD03

G

- Fra1
- Egr1
- Fos
responses of FGF/MEK/ERK signaling. Because SB43 can substitute for PD03 to maintain the pluripotency of ES cells and because the expression of Fgf genes could be down-regulated by SB43 in J1 ES cells (22), we asked whether TGF-β signaling could modulate FGF/MEK/ERK signaling activity. LSC-ES cells that were cultured in N2B27/LIF medium overnight were treated with 10 μM SB43, and p-ERK levels were examined using Western blots. Indeed, p-ERK1/2 was decreased significantly in ES cells treated with SB43 for 8 h or longer (Fig. 5C). The BMP pathway can suppress FGF/MEK/ERK signaling through Smad1/5/8 in mouse ES cells (44). Therefore, the levels of phosphorylate Smad1 (p-Smad1), an indicator of BMP signaling activity, were detected. SB43 could not enhance the level of p-Smad1 (Fig. 5C), suggesting that the inhibition of ERK activity by SB43 is independent of BMP signaling.

Next we asked whether the negative regulation of FGF/MEK/ERK signaling could be relieved by the withdrawal of SB43. LSC-ES cells were washed twice with N2B27 and cultured in N2B27/LIF/CHIR medium. Cells were harvested for immunoblotting 0, 0.5, 2, 4, 8, 12, and 24 h after removing SB43. The p-ERK1/2 level was increased significantly after 4 h (Fig. 5D). In addition, when Activin A (30 ng/ml) was added to the culture for 12 or 24 h, the ERK activities were enhanced further (Fig. 5D). Consistently, the expression of the FGF/ERK pathway downstream targets Egr1 and Fra1 were up-regulated by SB43 withdrawal and Activin A addition (Fig. 5E). Therefore, in the presence of LIF and CHIR, TGF-β signaling can regulate ERK activity.

To investigate the long-term effect of SB43 on the regulation of the FGF/MEK/ERK signaling pathway, we examined the levels of p-ERK in ES cells cultured in serum (serum/LIF), LIF/CHIR, LSC, or LPC for more than 10 passages. Compared with ES cells grown in serum or LIF/CHIR, the p-ERK level was decreased significantly in LSC-ES cells and could not be detected in LPC-ES cells (Fig. 5F). Similarly, the expression of ERK target genes was lower in cells grown under LSC conditions than in those grown with serum or LIF/CHIR medium but was relatively higher than that of cells grown in LPC medium (Fig. 5G). Taken together, these results suggest that SB43 inhibits the TGF-β signaling pathway to maintain the pluripotency of mouse ES cells by reducing ERK activity.

**DISCUSSION**

In this study, we show that SB43, a TGF-β signaling inhibitor, together with the histone deacetylase inhibitor VPA, could substitute for Oct4 to reprogram MEFs into iPSCs. In addition, the inhibition of TGF-β signaling by SB43 can sustain the pluripotency of iPSCs and ES cells. Furthermore, we revealed that TGF-β signaling inhibition regulates FGF/MEK/ERK signaling by reducing ERK phosphorylation in ES cells. Our results suggest that TGF-β signaling plays essential roles in the generation and maintenance of PSCs.

The transduction of Sox2, Oct4, Klf4, and c-Myc can reprogram somatic cells to pluripotent cells (6). Unfortunately because of the involvement of the viral vector and oncogenic factors in the system, induced pluripotent stem cells are not the best choice for clinical therapy (45, 46). Accordingly, small molecules that can substitute for reprogramming factors showed light on the medical applications of iPSCs. Sox2 and c-Myc, but not Oct4 or Klf4, can be replaced by SB43 in cells grown with serum (24, 25). Consistent with these reports, iPSCs could not be generated using SB43 or VPA alone in MEFs cultured under serum-free conditions and transduced with Sox2, Klf4, and c-Myc (Fig. 2). Interestingly, under serum-free conditions, SB43 in combination with VPA could substitute for Oct4 to reprogram MEFs to iPSCs (Fig. 2). VPA is likely involved in the conformation changes of the chromatin structure, which, in turn, reduces the reprogramming barriers to activate endogenous gene expression. As reported previously (37–39), we also observed that, in serum-free medium containing both SB43 and VPA, the expression of a few genes that can replace Oct4 during reprogramming was up-regulated (Fig. 2). iPSCs can be generated by using seven small molecules. Although VPA and the TGF-β signaling inhibitor 616452 were among these seven molecules, only the cyclic AMP activator Forskolin was thought to be a chemical substitute for Oct4 (47). In this study, our data indicate that the inhibition of TGF-β signaling by a small molecule, SB43, can substitute for Oct4 to reprogram MEFs into iPSCs.

TGF-β signaling plays important roles in the maintenance of mouse epiblast stem cells and human ES cells (48–50). However, the function of TGF-β signaling in the pluripotency of mouse ES cells has not been fully elucidated. Some studies have shown that the TGF-β pathway is involved in the proliferation and pluripotency of mouse ES cells (19–23), but others have suggested that the TGF-β pathway is not the key for mouse ES cell pluripotency (16, 18). The discrepancy may be caused by the different culture conditions. In serum-containing medium, the inhibition of TGF-β signaling by Smad7 or SB43 dramatically decreases the propagation but not the pluripotency of ES cells (16). In contrast, in serum-free medium, the presence of...
both the ERK inhibitor PD03 and SB43 not only generates ES cells from blastocysts but also maintains the pluripotency of the ES cells in the long term (23). We also observed that SB43 could maintain the expression of pluripotency markers (Fig. 5) but could not maintain the long-term propagation of ES cells in the absence of CHIR (data not shown). However, in the presence of CHIR, SB43 can sustain mouse ES cells and iPSCs for many passages (Fig. 3). These observations suggest that ES cells cultured under different culture conditions may respond differently to TGF-β signaling.

FGF is needed for later lineage segregation (12, 26, 51, 52). The repression of FGF/MEK/ERK signaling likely maintains ES cells at a state before the lineage commitment. In contrast, activation of the ERK pathway by FGF4 or serum generates more restricted and heterogeneous cell populations from ES cells cultured in the serum/LIF medium (36, 43). Therefore, inhibition of FGF/MEK/ERK signaling is critical for the generation of homogeneous ES cells. In this study, we introduced the TGF-β signaling inhibitor SB43 to replace the FGF/ERK signaling inhibitor PD03 under traditional LIF/PD03/CHIR conditions and found a novel culture condition for mouse iPSCs and ES cells (Fig. 3). ERK activity was inhibited under both the LIF/PD03/CHIR and the LIF/SB43/CHIR conditions (Fig. 5). ES cells cultured under LIF/SB43/CHIR conditions preserved multiple features of ES cells cultured under LIF/PD03/CHIR conditions, including minimal expression of lineage genes, uniform expression of the pluripotency marker Nanog, and similar differentiation capability (Figs. 3 and 4). These results suggest that the nature of pluripotency obtained by the two different approaches may be similar.

In conclusion, LIF/SB43/CHIR may provide a proper environment for the generation and maintenance of PSCs that is similar to the effect of LIF/PD03/CHIR. Clearly, extracellular signals that are involved in the ESC pluripotency can be regulated by small molecules. Investigations into the mechanisms through which the small molecules can substitute for any reprogramming gene to generate and maintain iPSCs would benefit the clinic applications of these cells.

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