

Generation of iPSCs from mouse fibroblasts with a single gene, *Oct4*, and small molecules

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The introduction of four transcription factors *Oct4*, *Klf4*, *Sox2* and *c-Myc* by viral transduction can induce reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), but the use of iPSCs is hindered by the use of viral delivery systems. Chemical-induced reprogramming offers a novel approach to generating iPSCs without any viral vector-based genetic modification. Previous reports showed that several small molecules could replace some of the reprogramming factors although at least two transcription factors, *Oct4* and *Klf4*, are still required to generate iPSCs from mouse embryonic fibroblasts. Here, we identify a specific chemical combination, which is sufficient to permit reprogramming from mouse embryonic and adult fibroblasts in the presence of a single transcription factor, *Oct4*, within 20 days, replacing *Sox2*, *Klf4* and *c-Myc*. The iPSCs generated using this treatment resembled mouse embryonic stem cells in terms of global gene expression profile, epigenetic status and pluripotency both *in vitro* and *in vivo*. We also found that 8 days of *Oct4* induction was sufficient to enable *Oct4*-induced reprogramming in the presence of the small molecules, which suggests that reprogramming was initiated within the first 8 days and was independent of continuous exogenous *Oct4* expression. These discoveries will aid in the future generation of iPSCs without genetic modification, as well as elucidating the molecular mechanisms that underlie the reprogramming process.

Keywords: pluripotency; iPS cells; oct4; reprogramming

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Introduction

The introduction of four transcription factors, *Oct4*, *Klf4*, *Sox2* and *c-Myc*, by viral transduction can induce the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), which resemble embryonic stem cells (ESCs) [1-4]. The iPS technique represents a breakthrough in the stem cell field and provides a promising cell resource for customized patient-specific cell therapies. However, the clinical applications of iPSCs

are hindered by the potential risks of genetic mutation caused by the integration of exogenous genetic material into chromosomes. Although several nonintegrative methods have been developed to generate iPSCs, induction efficiency is still quite low [5-8].

However, recent reports indicate that the reprogramming efficiency can be enhanced by the presence of small molecules, such as valproic acid (VPA, an HDAC inhibitor), AZA (a DNMT inhibitor), butyrate (an HDAC inhibitor) and vitamin C [9-12]. In addition, two small molecule inhibitors, CHIR99021 (a GSK3- β inhibitor) and PD0325901 (a MEK/ERK inhibitor), were found to enhance the completion and efficiency of reprogramming process [13].

Importantly, some small molecules have also been reported to be able to replace some transcription factors in iPSC generation. For example, a G9a inhibitor, BIX01294, was reported to induce iPSCs from neural

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stem cells, in place of *Oct4* [14]. Kenpaullone could substitute for *Klf4*, although the underlying mechanism is still unclear [15]. In addition, a transforming growth factor (TGF)- β inhibitor (named 616452) could replace *Sox2* during iPSC generation [16–18]. So far, at least two transcription factors, *Oct4* and *Klf4*, are still required to generate iPSCs from fibroblasts in the presence of a TGF- β receptor inhibitor [16, 17]. Thus, it became of intense interest to investigate whether the requirement of exogenous transcription factors could be further eliminated to achieve complete chemical reprogramming by novel small molecules or novel combinations of small molecules that facilitate reprogramming.

In this work, we found that a specific small molecule combination alleviated the requirement for *Sox2*, *Klf4* and *c-Myc* and induced mouse fibroblasts into iPSCs in the presence of a single transcription factor, *Oct4*. Our finding takes one step closer to the generation of iPSCs by small molecules without any genetic modification, and provides a unique platform for future screening to identify small molecules that could further replace the requirement for exogenous expression of *Oct4*.

Results

Generation of iPSCs with Oct4 and chemical combinations

In our initial experiments, we isolated OG-MEFs (E13.5 mouse embryonic fibroblasts (MEFs) from OG transgenic mice, which contain an *Oct4*-GFP reporter system to indicate the pluripotent status). OG-MEFs were transduced with lentiviral vectors expressing *Oct4/Sox2/Klf4* and cultured in the presence of several selected small molecules reported to facilitate reprogramming. We found that small molecules VPA and CHIR99021 greatly improved the efficiency of GFP+/iPS-like colony generation (approximately 30-fold) so that approximately 30 iPS colonies were generated from 1×10^4 MEFs within 15 days after infection. Experiments were repeated three times and representative data are shown in Figure 1A.

Next, we used a TGF- β inhibitor, 616452, which reportedly can replace *Sox2* during iPSC generation [16, 17]. We first found that iPSCs were efficiently generated using only two transcription factors *Oct4* and *Klf4*, in combination with VPA, CHIR99021 and 616452 (this small molecule combination was termed VC6); 5–20 GFP+/iPS-like colonies were generated from 5×10^4 MEFs within 15 days after infection. Experiments were repeated three times and representative data are shown in Figure 1B. We further discovered that GFP+/iPS-like colonies were generated using only *Oct4* and VC6 treatment when MEFs and adult fibroblasts (isolated from the

lips of a 4-week-old mouse) were cultured for 30 days, though the efficiency was quite low, only 1 in 2×10^5 cells (in five experiments, two could not obtain GFP+/iPS-like colonies) (Figure 1D). To confirm the necessity of the small molecules, these small molecules were each removed in turn, from the *Oct4*-induced reprogramming protocol. iPSCs could not be obtained in the absence of VPA, CHIR 99021 or 616452.

To improve reprogramming efficiency, small molecule libraries (Tocris Total; Sigma LOPAC and Prestwick Chemical Libraries) were screened in combination with exogenous *Oct4/Sox2/Klf4* in MEFs to identify the compound candidates that facilitate reprogramming. We found that an H3K4 demethylation inhibitor, tranylcypromine, significantly promoted iPSC generation (by approximately 20-fold) induced by *Oct4/Sox2/Klf4* with a level of efficiency similar to that with the use of VPA. When VPA and tranylcypromine were added together, iPSC generation efficiency increased further. Representative data from three experiments are shown in Figure 1C. Next, we found that when tranylcypromine was added to the VC6 chemical combination (termed VC6T), approximately 1–15 GFP+/iPS-like colonies (in 10 independent experiments) were generated from 5×10^4 OG-MEFs on day 18 following transduction of *Oct4* alone (Figure 1E and 1F). The reprogramming efficiency is much improved compared to the MEFs transduced with *Oct4* and treated with VC6. In contrast, no colonies appeared in control OG-MEFs without small molecule treatment or without *Oct4* introduction. GFP+ colonies were picked and passaged, and PCR analysis confirmed the presence of only exogenous *Oct4* DNA in the genome, without any exogenous *Klf4*, *Sox2* and *c-Myc* (Figure 1G). Similar results were obtained with three batches of OG-MEFs and from MEFs of different mouse strains, ICR \times OG, 129 \times OG and C57 \times OG (data now shown). In addition, we tested different concentrations of small molecules in VC6T, and suggest the optimal concentrations in Supplementary information, Figure S1.

Pluripotency and differentiation characteristics of iPSCs generated with Oct4 and chemical combinations

GFP+/iPS-like colonies generated from OG-MEFs were selected, replated onto MEF feeder cells and expanded under mouse embryonic stem cell (mESC) growth conditions without additional small molecule treatment. These GFP+/iPS-like cells (referred to as *Oct4*-iPSCs) had normal karyotypes and maintained GFP+/iPS-like morphology and alkaline phosphatase (AP) activity for more than 20 passages (Figure 2A). The pluripotent characteristics of the *Oct4*-iPSCs were further examined by immunostaining and reverse-tran-

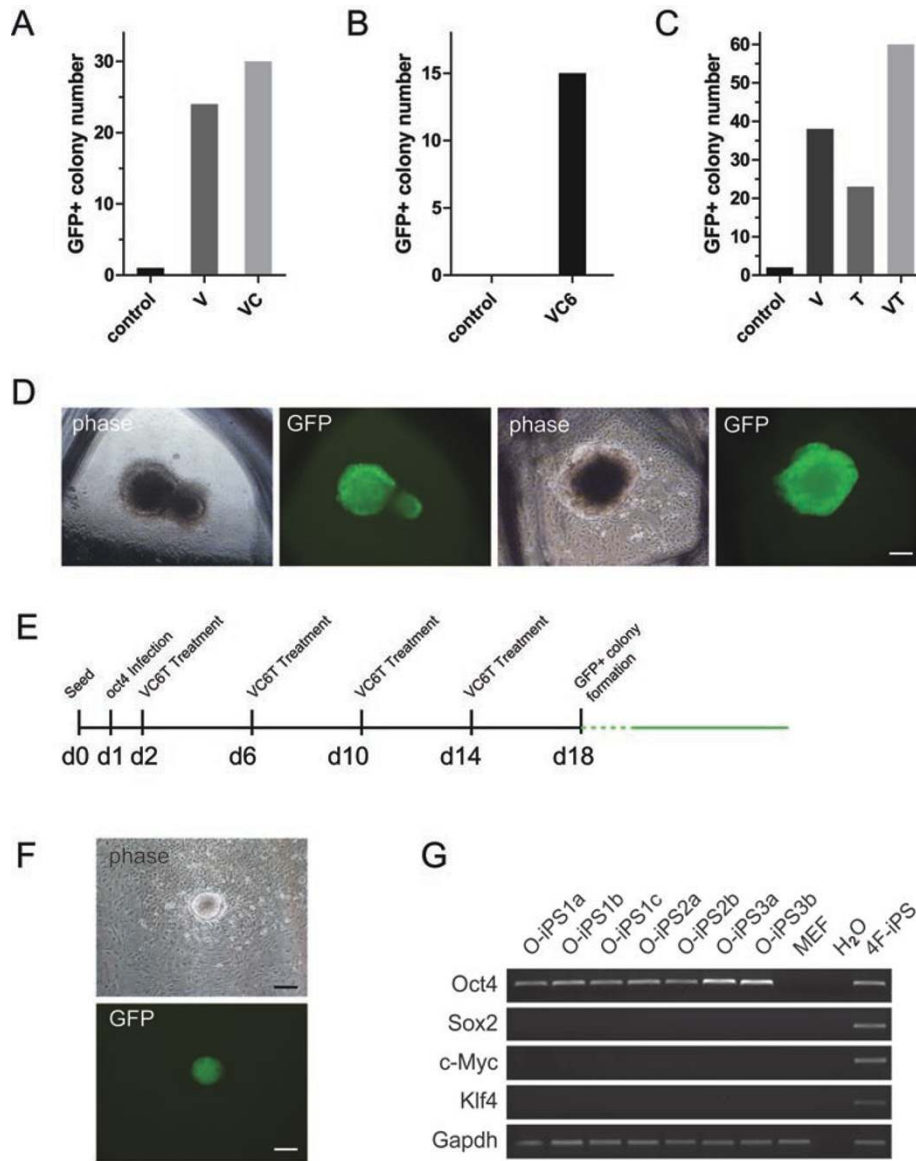


Figure 1 Generation of iPSCs from fibroblasts by *Oct4* and small molecules. **(A)** GFP+/iPS-like colony numbers induced from OG-MEFs transduced with *Oct4/Sox2/Klf4*. VPA and CHIR99021 greatly improved the efficiency of GFP+/iPS-like colony generation (approximately 30-fold). Approximately 30 iPS colonies were generated from 1×10^4 MEFs at day 15 after infection. Similar results were obtained in three independent experiments. “V” stands for VPA. “VC” stands for the combination of VPA and CHIR99021. **(B)** GFP+/iPS-like colony numbers induced from OG-MEFs transduced with *Oct4/Klf4*, in combination with VPA, CHIR99021 and 616452 (VC6). Approximately 5-20 GFP+/iPS-like colonies were generated from 5×10^4 MEFs at day 15 after infection. Similar results were obtained in three independent experiments. **(C)** GFP+/iPS-like colony numbers induced from OG-MEFs transduced with *Oct4/Sox2/Klf4*. Tranylcypromine significantly promoted iPS generation (approximately 20-fold), with an efficiency similar to that of VPA. When VPA and tranylcypromine were added together (VT), iPS generation efficiency was further increased. The number of iPS colonies generated from 1×10^4 MEFs was counted on day 15 after infection. Similar results were obtained in three independent experiments. “V” stands for VPA. “T” stands for tranylcypromine. **(D)** A typical GFP+/iPS-like colony generated from MEFs (left) and adult fibroblasts (right) after 30 days of *Oct4* and VC6 treatment. **(E)** Timeline of *Oct4*-iPSC generation using one single factor *Oct4* and small molecule treatment (VC6T: VPA, CHIR99021, 616452 and tranylcypromine). Culture medium containing small molecules was changed every four days. **(F)** GFP+ colonies appeared 18 days after OG-MEFs were transduced with *Oct4* and treated with VC6T. Bars, 500 μ m. **(G)** Genome PCR showed that these *Oct4*-iPSCs had only the exogenous *Oct4* insertion and were free of other exogenous factors (1a,1b,1c: *Oct4*-iPSC lines from MEFs of ICR \times OG genetic background; 2a,2b: *Oct4*-iPSC lines from MEFs of 129 \times OG genetic background; 3a,3b: *Oct4*-iPSC lines from MEFs of C57 \times OG genetic background; 4f-iPS: iPSCs induced by the original four transcriptional factors, *Oct4*, *Sox2*, *Klf4* and *c-Myc* as positive control).

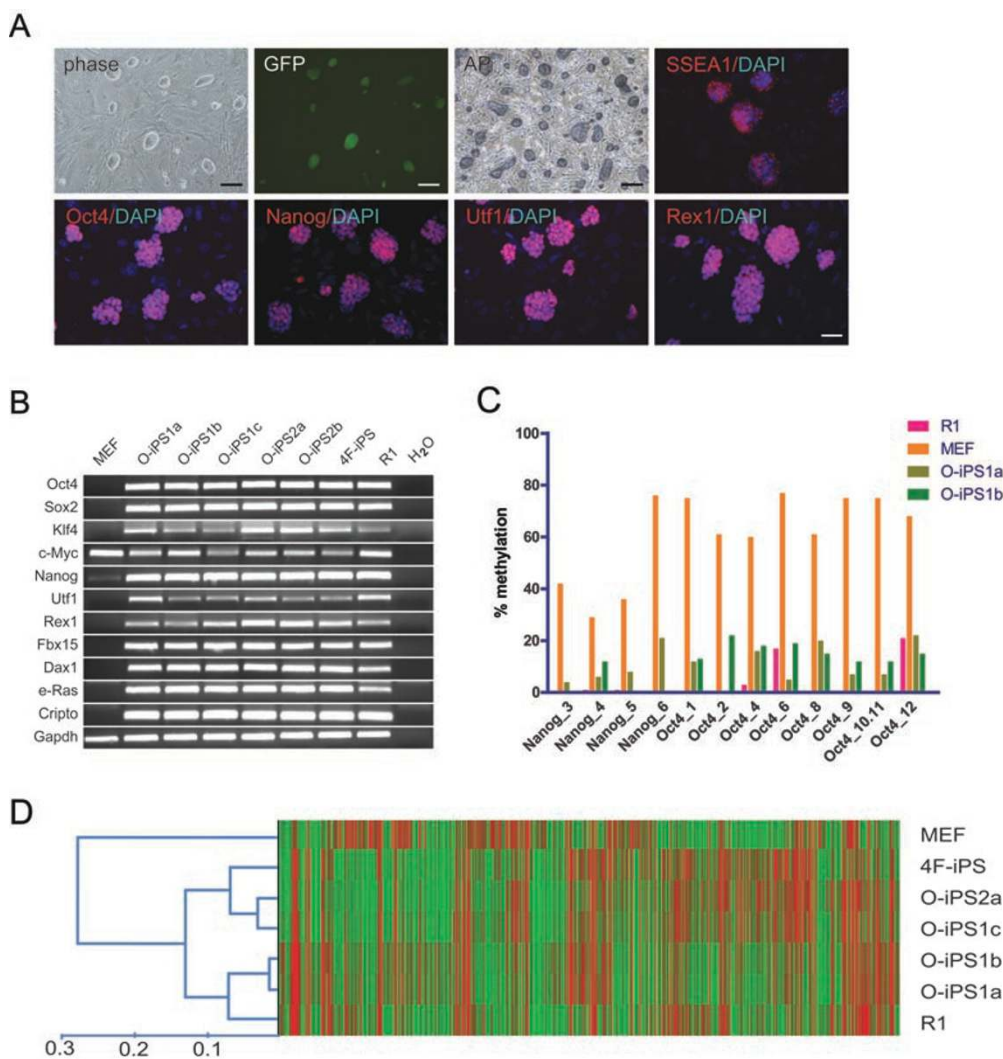


Figure 2 *Oct4* induced iPSCs to express pluripotency markers and resemble ESCs. *Oct4*-induced iPSCs maintained ESC-like morphology, AP activity and GFP expression in mESC growth media (bars, 500 μ m), while expressing pluripotency markers Oct4, Nanog, Utf1, Rex1 and SSEA1, detected by immunofluorescence (bars, 100 μ m). **(A)** RT-PCR analysis showed the pluripotent marker gene expression of these *Oct4*-iPSCs (1a,1b,1c: three separate *Oct4*-iPSC lines from MEFs of ICR \times OG genetic background; 3a,3b: two separate *Oct4*-iPSC lines from MEFs of 129 \times OG genetic background). **(B)** DNA methylation analysis of several CpG sites in the Nanog and *Oct4* promoters, indicating that the demethylation of Nanog and *Oct4* promoters in *Oct4*-iPSCs was similar to that of mESCs. Nanog and *Oct4* promoters of MEFs were hypermethylated. **(C)** Microarray analyses showed a similar global gene expression profile among *Oct4*-iPSCs, 4F-iPSCs and mESCs (R1), which were quite different from that of OG-MEFs.

scription (RT)-PCR. Specifically, *Oct4*-iPSCs were positively stained for pluripotency-specific mESC markers, including Oct4, Sox2, Nanog, Utf1, Rex1 and SSEA1 (Figure 2A). RT-PCR showed that the iPSCs also expressed pluripotency marker genes, such as *Nanog*, *Utf1*, *Rex1*, *Fbx15*, *Dax1*, *e-Ras* and *Cripto* (Figure 2B). DNA methylation analysis of the *Nanog* and *Oct4* promoters showed that demethylation levels in *Oct4*-iPSCs were similar to those of mESCs. In contrast, the *Nanog* and

Oct4 promoters of normal OG-MEFs were hypermethylated (Figure 2C). Microarray analyses showed similar global gene expression profiles among *Oct4*-iPSCs, 4F-iPSCs (iPSCs induced by *Oct4*, *Sox2*, *Klf4* and *c-Myc*) and mESCs, which were very different from that of OG-MEFs (Figure 2D). These results indicate that *Oct4*-iPSCs share characteristics with 4F-iPSCs and mESCs.

To analyze the differentiation potential of the *Oct4*-iPSC lines, we tested their capacity to differentiate into cell

types of the three germ layers. All three *Oct4*-iPSC lines chosen for teratoma testing formed teratomas *in vivo* 4-6 weeks after injection, and tissues of all three germ layers were detected (Figure 3A). In addition, these *Oct4*-iPSCs were efficiently incorporated into the inner cell masses of mouse blastocysts after aggregation with eight-cell em-

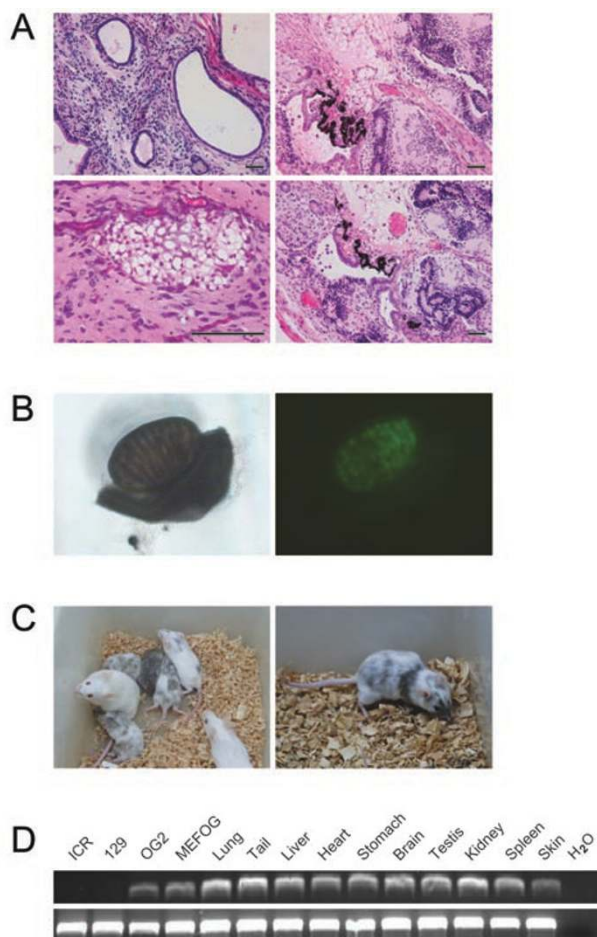


Figure 3 Differentiation potential of *Oct4*-iPSCs. **(A)** Teratoma formation of *Oct4*-iPSCs. The tissues of all three germ layers, such as pigmented retinal epithelium, cartilage, neural tube-like epithelium and gut-like epithelium, were detected in these *Oct4*-iPSC-derived teratoma sections. Bars, 200 μ m. **(B)** When the *Oct4*-iPSC aggregated embryos were transplanted into mice, GFP+ cells were detected in the gonad tissues of 17 days post copulation embryos, which suggests that the iPSC cell contribution to the germ line. **(C)** Adult mice with a high degree of chimerism were developed from *Oct4*-iPSC lines generated from MEFs (left) and adult fibroblasts (right), after blastocyst transplantation. **(D)** Genome PCR showed that these *Oct4*-iPSCs contributed to brain, lung, tail, liver, heart, stomach, testis, kidney, spleen and skin in a chimeric mouse. Tail fibroblasts from ICR and 129 mice were used as negative control. Tail fibroblasts from OG2 mice and MEFOG, which have p*Oct4*-GFP alleles in their genomes, were used as positive control.

bryos. When the aggregated embryos were transplanted into mice, GFP+ cells were detected in the gonadal tissues at 17 days post coitum (dpc), suggesting that *Oct4*-iPSCs contribute to the germ line (Figure 3B). Chimeric embryos further developed into adult mice with a high degree of chimerism (Figure 3C and 3D). These results indicate that the *Oct4*-iPSC lines can develop and differentiate *in vivo* to generate chimeric mice with germ-line contribution. Thus, *Oct4*-iPSCs have similar differentiation potential to primary mouse ESC.

We further tested whether the VC6T small molecule combination could enable *Oct4*-induced reprogramming in adult mouse fibroblasts. Dermal fibroblasts were isolated from the lip and back of 8-week-old mice. We found that *Oct4* in combination with VC6T treatment indeed induced reprogramming of adult mouse fibroblasts, although the induction time was longer than that of MEFs (more than 25 days after transduction) (Supplementary information, Figure S2A). The resulting adult iPSCs expressed the pluripotency markers *Oct4*, *Nanog*, *Utf1*, *Rex1* and *SSEA1*, as detected by immunofluorescence (Supplementary information, Figure S2B). *Oct4*-iPSCs generated from adult fibroblasts were able to produce adult chimaeras, after blastocyst transplantation (Figure 3C). We also confirmed by genome PCR that iPSCs derived from adult mouse fibroblasts had only one introduced reprogramming gene, *Oct4* (Supplementary information, Figure S2C).

Reprogramming kinetics of mouse fibroblasts by DOX-inducible *Oct4* expression system and small molecules

To better understand the mechanism of the reprogramming process in *Oct4*-induced iPSC generation, we established a tet-on system to drive *Oct4* expression in MEFs (TET-O MEFs). MEFs were treated with VC6T throughout the reprogramming process, and doxycycline (dox) was added at different time points over the course of the experiment. We found that 8 days of dox-induced *Oct4* expression was sufficient for iPSC generation observed at day 24 after transduction (Figure 4A). Efficiency increased when *Oct4* expression was induced for 12 days (Figure 4A), which is in consistent with previous reports that a greater number of iPSCs are generated when exogenous reprogramming factors were expressed for longer period of time [19, 20]. However, iPSC colony numbers decreased when *Oct4* was induced for more than 12 days, and most of the iPSC colonies emerged 4-8 days after dox withdrawal (Figure 4A). These data suggest that expression of exogenous *Oct4* must be silenced to facilitate the activation of endogenous pluripotency transcriptional circuitry, which is consistent with previous reports that reprogramming efficiency may be im-

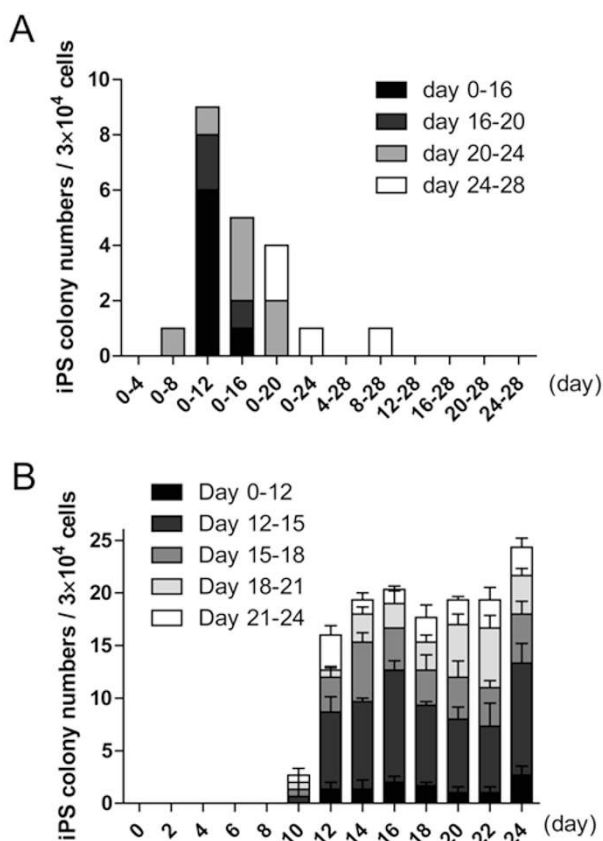


Figure 4 The efficiency of iPSC generation with DOX-inducible *Oct4* expression and VC6T treatment. **(A)** iPSC colony numbers generated by different induction time of *Oct4* using the tet-on system. Doxycycline (dox) was added at different time periods during the reprogramming process as shown on the x axis. The various shaded bars indicate the days on which colonies appeared in the culture. Similar results were obtained in three independent experiments. **(B)** iPSC colony numbers generated by different treatment times. The numbers on x axis stand for the days with VC6T treatment from day 0 after transduction. Dox was added during the entire process to induce *Oct4* expression. Colony numbers were calculated at days 12, 15, 18, 21 and 24, shown by various shaded bars. Results were obtained in three independent experiments.

paired with the prolonged expression of exogenous genes [19]. The results suggest that *Oct4*, together with VC6T treatment, initiates the reprogramming process early within the first 8 days. After that, *Oct4* is not essential to the reprogramming process, but it may enhance the efficiency of iPSC generation from days 8 to 12, whereas exogenous *Oct4* may impair iPSC generation after day 12.

We next induced *Oct4* expression throughout the reprogramming process, and added VC6T at different time points. VC6T treatment in the first 10 days was sufficient

to enable *Oct4*-induced iPSC generation (Figure 4B). These results are consistent with our findings that endogenous *Sox2* and *Nanog* were expressed and that *Klf4* expression was elevated at days 10-15 after transduction, before the emergence of iPSCs (Supplementary information, Figure S3). Endogenous expression of *Oct4* was not detectable before the generation of iPSCs, however. It is possible that endogenous expression of *Sox2*, *Klf4* and *Nanog* triggered by the small molecules help drive the reprogramming process in *Oct4*-induced iPSCs.

Discussion

In this study, we found that the combination of four small molecules, VPA, tranilcypromine, CHIR99021 and 616452 (VC6T), was sufficient to induce reprogramming in combination with a single transcription factor, *Oct4*, thus replacing *Sox2*, *Klf4* and *c-Myc*. Furthermore, the resulting *Oct4*-iPSCs exhibited differentiation potential into cell types of all three germ layers and germ-line transmission in chimeric mice.

Oct4 is the master regulatory gene in cell pluripotency and may serve as a pluripotency determinant in reprogramming [21, 22]. On the bases of the data shown here, we propose that the small molecule combination VC6T may facilitate iPSC generation by lowering several major barriers to the reprogramming process. VPA (an HDAC inhibitor) and tranilcypromine (an H3K4 demethylation inhibitor) are epigenetic modulators that have been reported to facilitate iPSC generation [9, 23, 24]. VPA was previously found to cause upregulation of the expression of ESC-specific genes in MEFs [9]. Tranilcypromine was also reported to activate endogenous *Oct4* expression in EC cells [25]. The effects of these two small molecules suggest that H3K4 demethylation and histone deacetylation could be two critical epigenetic barriers to reprogramming that may repress the establishment of a pluripotency transcriptional network. In addition, it has been reported that either GSK3- β inhibition by CHIR99021 or TGF- β signaling inhibition by 616452 could efficiently replace *Sox2* for reprogramming [16, 17, 24]. Inhibition of GSK3- β by Wnt signaling has been reported to enhance mESC self-renewal and cell reprogramming, possibly by regulating the stability of *c-Myc* protein [26, 27]. In addition, TGF- β inhibition was reported to promote mesenchymal-to-epithelial transition [28-30] and facilitate *Nanog* gene expression during the reprogramming process [17]. Taken together with our findings, GSK3- β and TGF- β signaling may be two major barriers that normally repress the reprogramming process. In our study, lowering these four major reprogramming barriers (two epigenetic barriers and two signaling

barriers) was sufficient to allow reprogramming by *Oct4* induction alone.

Consistent with this model, we found that VC6T treatment facilitated *Sox2*, *Klf4* and *Nanog* (SKN) expression in *Oct4*-induced reprogramming. However, it is possible that the elevated expression of SKN represents only tangential markers of an *Oct4*-induced reprogramming process, because *Oct4* was not strictly required 8 days after infection. Alternatively, SKN expression may have been activated earlier in a very small proportion of MEF cells, which could account for the *Oct4*-induced reprogramming but may not be detectable by RT-PCR. Therefore, it is still unclear whether elevated expression of SKN accounts for the mechanism of VC6T-facilitated reprogramming. More experiments are required to elucidate the mechanism of the iPSC induction process.

One small molecule, Kenpaullone, has been recently reported to be able to replace the reprogramming factor *Klf4* in the generation of iPSCs from MEFs [15]. However, the other three factors, *Oct4*, *Sox2* and *c-Myc*, were still required. In this study, we replaced *Klf4* with small molecules and enabled MEF reprogramming with only one single factor, *Oct4*. Neural stem cells have been reported to be reprogrammed into pluripotency by *Oct4* transduction alone, although these cells endogenously express *Sox2* and share a similar expression pattern of several pluripotency markers, including SSEA-1 and ALP, with ESCs [31]. The MEFs used in our study did not endogenously express *Sox2*, and their gene expression profile differs extensively from that of ESCs. Moreover, the MEFs could not be reprogrammed with *Oct4* in the absence of additional small molecule treatment.

The generation of *Oct4*-iPSCs from MEFs reported here represents an important step toward identifying a chemical combination that can entirely replace exogenous reprogramming factors, as we have identified a combination of four small molecules that allows reprogramming in the presence of a single exogenous transcription factor. Based on these findings, a screen assay could be designed to identify other small molecules that could replace *Oct4*, in combination with the small molecules identified here. These advances will be beneficial for elucidating the underlying molecular mechanisms of reprogramming and for generating iPSCs without genetic modifications that may hamper their clinical applications.

Materials and Methods

Mice

The transgenic mouse strain TgN(GOFGFP)11Imeg (OG) were bought from RIKEN BioResource Center. Other mouse strains included ICR, 129S2/SvPasCrIVr (129) and C57BL/6NCrIVr (C57), which were all bought from Beijing Vital River Laboratory Animal

Inc. Animal experiments were performed according to the Animal Protection Guidelines of Peking University, China.

Cell culture

Primary MEFs were derived from transgenic mice including ICR×OG, 129×OG and C57×OG. Adult skin fibroblasts were isolated from the lips and backs of 7-week-old transgenic mice including ICR×OG, 129×OG and C57×OG. Fibroblasts and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 10% fetal bovine serum (Invitrogen). mESC line R1 and iPS cells were maintained on Mitomycin C-treated MEFs in mESC culture medium consisting of 80% DMEM/F12 (Invitrogen), 10% knockout serum replacement (Invitrogen), 10% fetal bovine serum, 1 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol (all from Invitrogen) and 1 000 U/ml Lif (Millipore). iPSCs and mESCs were passaged by trypsin (Invitrogen). The medium was changed every day.

Lentivirus infection and iPSC cell generation

Lentiviral vectors containing the four reprogramming factors (OCT4, SOX2, KLF4 and c-MYC) were described in the previous reports [32]. Lentivirus production, collection and infection were also the same as described [32]. For tet-on Oct4 systems, Fu-tet- hOct4 and FUDeltaGW-rtTA [19] were co-transfected into fibroblasts. Dox were added by 1 μg/ml. The culture medium of the infected cells was changed into mESC culture medium 48 h after infection. Although ascorbic acid (vitamin C) is contained in knockout serum replacement, additional 10 ng/ml stabilized form of ascorbic acid (Sigma) was added into mESC culture medium for iPSC generation [11]. To promote fibroblasts proliferation, 10 ng/ml basic fibroblast growth factor (P&A Biotech) was added during the first 8 days after infection. The medium with small molecules VPA (0.5mM; Sigma), CHIR99021 (3 μM; Stemgent), 616452 (1 μM; Calbiochem) and tranlycypromine (5 μM; Tocris) was changed every 4 days. The GFP+/iPS-like colonies were picked at around day 20 to day 30 after viral transduction and maintained in the culture condition for mESCs.

Alkaline phosphatase staining and immunofluorescence

To detect AP activity, we washed the cells with phosphate-buffered saline three times and stained with BCIP/NBT (Promega) for 15 min. For immunofluorescence, the primary antibodies included SSEA-1 (1:200; Millipore), Oct4 (1:200; Abcam), Sox2 (1:200; Santa Cruz), Rex1 (1:200; Santa Cruz), Nanog (1:40; R&D), Utl1 (1:200; Abcam), MAP2 (1:100; Millipore), α-SMA (1:200; Santa Cruz) and ALB (1:100; Dako). Secondary antibodies were rhodamine-labeled donkey anti-mouse IgG, rhodamine-labeled donkey anti-rabbit IgG, rhodamine-labeled donkey anti-goat IgG, rhodamine-labeled donkey anti-goat IgM and rhodamine-labeled goat anti-mouse IgG3 (all from Santa Cruz, 1:100 dilution). 4'-Diamidino-2-phenyl indole was used for nuclear staining.

DNA methylation analysis

Genomic DNA sodium bisulfite conversion was performed using EZ-96 DNA Methylation kit (Zymo Research, Orange County, CA, USA). Sequenom's MassARRAY platform was used to perform quantitative methylation analysis, as described [31]. PCR primers were designed using EpiDESIGNER (<http://www.epide->

signer.com).

RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen) and reverse transcribed using EasyScript Reverse Transcriptase (TransGen Biotech) according to the manufacturer's protocol. PCR amplification of different genes was performed using 2× EasyTaq SuperMix (TransGen Biotech). The primers used are given in Supplementary information, Table S1.

In vitro differentiation and teratoma

For embryoid body formation, we cultured iPS cells in an uncoated cell culture suspension dish (Corning) in the presence of DMEM supplemented with 15% fetal bovine serum, 1 mM L-glutamine, 1% nonessential amino acids and 0.1 mM β-mercaptoethanol (all from Invitrogen). Embryoid bodies cultured in suspension for 7 days were transferred to plates coated with 0.1% gelatin (Sigma). Attached cells were cultured for 6–9 days, and then used for further analysis. For neural cell differentiation, the 4-day embryoid bodies were plated in chemical-defined medium (DMEM/F12 with N2B27 supplement; Invitrogen) on Matrigel-coated plates. The medium was changed every day. For teratoma formation *in vivo*, per 10⁵ iPS cells were injected into the kidney capsule of one severe combined immunodeficient beige mouse (*n* = 5). Teratomas were recovered 4 weeks after grafting. Control mice were injected with 1 million MEFs and failed to form teratoma (*n* = 3). Then they were embedded in paraffin and processed with hematoxylin and eosin staining.

DNA microarray

Total mRNA from MEF, iPSCs and mESCs (R1) were labeled with Cy5, hybridized to a mouse Oligo Microarray (Phalanx Mouse Whole Genome OneArray; Phalanx Biotech) according to the manufacturer's protocol. Three technical repetitions were performed. The median of each sample was used for hierarchical clustering. Complete-linkage hierarchical clustering was performed using cluster 3.0 (written by Michael Eisen, Stanford University) with Spearman's rank correlation coefficient as gene distances measurement and Pearson's correlation coefficient as sample distances measurement.

Chimera generation and genome PCR for tissue contribution

In brief, 8–10 iPS cells were microinjected into the eight-cell embryos using piezo-actuated microinjection pipette. After culturing for 1 day, the embryos were transferred into the uterine horns of a 2.5 dpc ICR pseudopregnant recipient for the development of chimera mice. To test the tissue contribution of the iPS cells into the chimeric mice, we performed genome PCR for pOct4-GFP allele, followed the protocols provide by RIKEN BRC. Primers used were OCGOFU35, CTAGGTGAGCCGTCTTTCCA; and EGFPUS23, TTCAGGGTCAGCTTGCCGTA.

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