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# Lithium, an anti-psychotic drug, greatly enhances the generation of induced pluripotent stem cells

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**Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by defined factors. The low efficiency of reprogramming and genomic integration of oncogenes and viral vectors limited the potential application of iPSCs. Here we report that Lithium (Li), a drug used to treat mood disorders, greatly enhances iPSC generation from both mouse embryonic fibroblast and human umbilical vein endothelial cells. Li facilitates iPSC generation with one (Oct4) or two factors (OS or OK). The effect of Li on promoting reprogramming only partially depends on its major target GSK3 $\beta$ . Unlike other GSK3 $\beta$  inhibitors, Li not only increases the expression of Nanog, but also enhances the transcriptional activity of Nanog. We also found that Li exerts its effect by promoting epigenetic modifications via downregulation of LSD1, a H3K4-specific histone demethylase. Knocking down LSD1 partially mimics Li's effect in enhancing reprogramming. Our results not only provide a straightforward method to improve the iPSC generation efficiency, but also identified a histone demethylase as a critical modulator for somatic cell reprogramming.**

**Keywords:** lithium; induced pluripotent stem cells; iPS; GSK3 $\beta$ ; Nanog; LSD1; histone demethylase

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## Introduction

The animal development from a fertilized egg to an individual is a programmed process and was believed to be irreversible in mammals. Recently the groundbreaking work demonstrated that ectopic expression of defined transcription factors (Oct4, Sox2, Klf4, c-Myc, Nanog, Lin28) could reprogram murine and human somatic cells to induced pluripotent stem cells (iPSCs) [1, 2]. Mouse iPSCs are similar to ESCs in most aspects and can develop into individuals after tetraploid complementation [3]. The iPSC technology has attracted enormous inter-

ests due to its potential biomedical applications. Patient-specific iPSCs could be created by reprogramming and they could be further differentiated into functional autologous cells for cell-based therapy without immunocompatibility issues and ethical concerns.

However, iPS cell applications are hindered by safety concerns due to the use of oncogenes and incorporation of viral DNA sequences. Many efforts have been taken to make iPSCs more amenable for therapeutic application, such as using reduced number of factors [4], non-integrating gene delivery approaches [5], or cell membrane permeable proteins to trigger the reprogramming [6]. However, the reprogramming efficiency is extremely low under these conditions.

Small molecules that can enhance the generation of iPSCs or compensate the requirement of certain oncogenic factors will be highly valuable. They can not only improve the reprogramming efficiency, but also help to

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dissect the underlying mechanisms. A number of chemicals have been reported to improve the reprogramming efficiency (reviewed by Li *et al.* [7]), including several epigenetic modulating agents, such as VPA, 5'-AZA and BIX-01294, and several major signaling pathway inhibitors, such as CHIR99021, PD0325901 and TGF $\beta$  receptor inhibitors. More recently, vitamin C (Vc) has been reported to greatly improve somatic cell reprogramming by alleviating cell senescence [8].

In our search for compounds that improve the efficiency of iPSC induction, we found that lithium (Li), a drug used to treat mood disorders, greatly enhances reprogramming in both mouse embryonic fibroblast (MEF) and human umbilical vein endothelial cells (HUVEC). Li also facilitates the generation of one factor (Oct4)-hiPSCs with combinations of other compounds. Several mechanisms, including GSK3 $\beta$  inhibition, enhanced Nanog expression and activation, and LSD1 downregulation, have been studied and demonstrated to play important roles in Li's enhancement of reprogramming.

## Results

### *Li promotes reprogramming of MEF cells*

We established a 96-well-plate-based chemical screening system for the four-factor (4F)-induced reprogramming (Figure 1A). During the screening, we found that treatment with the mood stabilizing drug lithium chloride (LiCl) [9] significantly increased the number of GFP<sup>+</sup> colonies. LiCl showed the greatest effect at 10 mM (Figure 1B). Li treatment not only increased the number of GFP<sup>+</sup> colonies, but also shortened the reprogramming process. At day 8, ~10 GFP<sup>+</sup> colonies could be observed in Li treated wells (5 000 MEF/well), while the control well had almost none (Figure 1C). At day 12, FACS analysis showed 10% of the cells being GFP<sup>+</sup> (Figure 1D). Similar enhancement of reprogramming was also observed with 3F (without c-Myc)-transduced MEF, though the process was slightly slower than 4F. At day 14, about 15 GFP<sup>+</sup> colonies could be observed in Li-treated wells. And the FACS data revealed a remarkable 14% cells being GFP<sup>+</sup> at day 16 (Figure 1J and 1K).

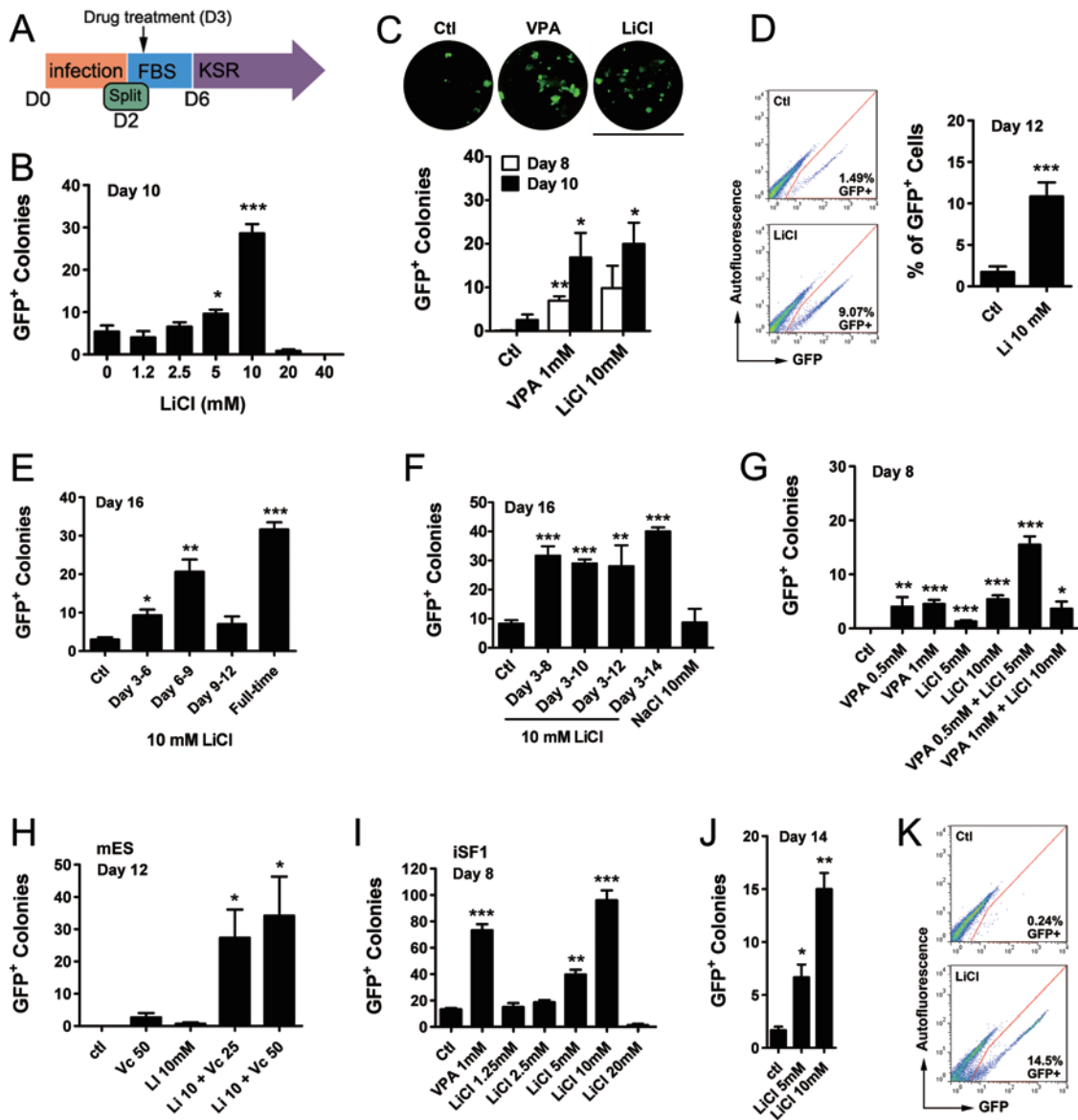
Li has been reported to regulate the proliferation of stem-like cells in retinoblastoma [10]. Chemicals that enhance the self-renewal of ES cells, such as PD0325901 and CHIR99021, have also been reported to enhance the generation of iPSC colonies [7]. To clarify whether Li facilitates the reprogramming process or enhances the proliferation of iPSCs after reprogramming, we treat the 4F-transduced MEF cells with LiCl for 72 h starting on day 3, 6, 9. We found that starting Li treatment on day 9 had no obvious effect on overall efficiency. In contrast, there

was a statistically significant 5- and 2.5-fold increase in the number of GFP<sup>+</sup> colonies in the cultures treated with Li starting on day 6 and 3, respectively (Figure 1E). We also treat the 4F-transduced MEF cells with LiCl for various durations starting from day 3. We found that the early stage of reprogramming (day 3-8) was most critical for the Li effects, as prolonged Li treatment did not further increase the efficiency (Figure 1F). In fact, prolonged treatment of Li caused reduction in colony size and eventual reduction in colony number (data not shown), indicating a cytotoxic effect. Therefore we decided that the treatment duration should be day 3-8. NaCl at 10 mM displayed no enhancement effect, indicating that Li is the effective component (Figure 1F). These data indicate that Li promotes the generation of iPSC colonies by facilitating the reprogramming process rather than enhancing the proliferation of iPSC cells.

Next we tested LiCl in combination with two reported reprogramming enhancers, VPA and Vc. The combination of LiCl and VPA displayed an additive effect (Figure 1G), suggesting that they act through different mechanisms. As the KSR supplement already contains Vc and additional Vc did not add effect to the overall reprogramming efficiency ([11] and our own observation), the combination of LiCl and Vc were tested in mES medium supplemented with FBS. The reprogramming process was much slower and efficiency was much lower in mES medium compared to KSR medium. At day 12, both Vc and LiCl showed marginal effect in enhancing reprogramming on their own. To our surprise, the combination of two displayed a robust synergistic effect (Figure 1H), suggesting crosstalk of pathways or targets regulated by these two small molecules. Recently, an optimized medium (iSF1) for mouse somatic cell reprogramming was reported [12], which uses KSR supplemented with 1/200 N2 and 5 ng/ml bFGF. Compared to our standard KSR medium, iSF1 significantly enhances the basal 4F-induced reprogramming efficiency. At day 8, more than 10 GFP<sup>+</sup> colonies could be observed from the control well, compared to almost zero in KSR medium (Figure 1I, 1C and 1G). Li still displayed a dose-dependent enhancement of reprogramming efficiency in iSF1 medium with maximal effect at 10 mM (~6-folds), and the fold of increase remains similar as in KSR medium (Figure 1I and 1B). This result indicates Li's beneficial effect does not overlap with N2 supplement and bFGF.

### *Cells reprogrammed with Li are pluripotent*

To test whether the enhanced reprogramming efficiency by Li could eventually result in *bona fide* iPSC cells, we generated a series of iPSC cell lines using the 4F or 3F plus LiCl methods by selecting the GFP<sup>+</sup> colonies.



**Figure 1** Li enhances the reprogramming efficiency of mouse fibroblasts. **(A)** Schematic representation of iPSC protocol with chemicals. **(B)** Dose-response of Li in 5 000 MEFs with 4F-infection. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(C)** Top: representative images of GFP<sup>+</sup> colonies in 96-well plates. The 4F-infected MEFs were treated with VPA (1 mM) or LiCl (10 mM). Cells were fixed at day 10. Scale bar: 6 mm. Bottom: GFP<sup>+</sup> colonies from 5 000 4F-infected MEFs in the presence of LiCl or VPA. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(D)** Left: representative FACS plots at day 12 from 4F-infected MEFs treated with LiCl (10 mM). Signal from the PE channel was used as a control for auto-fluorescence. Right: statistical data of GFP<sup>+</sup> cell percentage in 4F-infected MEFs treated with Li (10 mM) measured by FACS. Mean values  $\pm$  SEM of three independent experiments are shown. **(E)** Timing of Li action. LiCl (10 mM) was added for 3 days starting from day 3, 6 and 9 in 4F-infected MEFs and GFP<sup>+</sup> colonies were counted at day 16. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(F)** Treatment duration of Li. Starting from day 3, the 4F-infected MEFs were treated with 10 mM LiCl for various durations and GFP<sup>+</sup> colonies were counted at day 16. NaCl (10 mM) was added from day 3 to 14 as a negative control. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(G)** 4F-infected MEFs were treated with VPA, LiCl, or a combination of both. GFP<sup>+</sup> colonies were measured at day 8. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(H)** 4F-infected MEFs were treated with Vc, LiCl, or a combination of both in mES medium. GFP<sup>+</sup> colonies were measured at day 12. Mean values  $\pm$  SEM of three independent experiments are shown. **(I)** Li's effect in iSF1 medium. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(J)** Li's effect in 3F-infected MEF. GFP<sup>+</sup> colonies were measured at day 14. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . **(K)** Representative FACS plots at day 16 from 3F-infected MEFs treated with LiCl (10 mM). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , versus control.

qPCR analysis confirmed the reactivation and expression of the endogenous mouse Oct4, Sox2, Nanog and Rex1 (Figure 2A), and the silencing of viral genes (Figure 2B). PCR of genomic DNA of 3F-iPSCs confirmed the integration of retroviral Oct4, Sox2 and Klf4, but not c-Myc (Figure 2C). These iPSCs maintain GFP<sup>+</sup> and ES-like morphology. Immunocytochemistry revealed that they express typical pluripotency markers, such as alkaline phosphatase (AP), SSEA1 and Nanog (clone 4F 1-1 in Figure 2D and clone 3F-8 in Supplementary information, Figure S1A).

Karyotyping analysis revealed that the clone 4F 1-1 has normal 40, XX chromosomes (Figure 2E). Subcutaneous injection of the iPSCs into SCID mice led to teratoma formation in 3-4 weeks, containing tissues derived from all three germ layers, including epidermis structure (ectoderm), muscle and cartilage structure (mesoderm), and epithelium tube structure (endoderm) (Figure 2F and Supplementary information, Figure S1B). We next examined the ability of Li-induced iPSC cells to produce adult chimaeras. iPSC cells (clone 4F 1-1, 4F 4-1 and 3F-8) were injected into ICR-derived blastocysts, which were transplanted into the uteri of pseudopregnant mice. We obtained adult chimaeras from all three clones as determined by coat color (Figure 2G and Supplementary information, Table S2). We then crossed one of the chimaeras from clone 4F 1-1 with ICR male. Four of the total 16 F1 mice showed agouti coat color, confirming the germline transmission of iPSC clone 4F 1-1 (Figure 2G).

#### *Li enhances the generation of mouse and human iPSC cells with one or two factors*

Generation of mouse and human iPSC cells with reduced factors combined with small molecule compounds has been reported [13-15]. We tested Li (5 mM) in combination with VPA (0.5 mM) and RepSox (1  $\mu$ M) in OK-mediated reprogramming in MEF. At day 18, only one GFP<sup>+</sup> colony could be found out of 150 000 OK-transduced MEFs treated with VPA and RepSox. However, with Li, around 30 GFP<sup>+</sup> colonies were identified (Figure 3B). The cells treated with VPA and RepSox also developed colony-like structures, but most were not expressing Oct4-GFP (Figure 3A).

The chemical combinations that successfully facilitated the one factor (Oct4)-induced reprogramming in mouse and human cells are somewhat different. We were also interested to test whether positive effects of Li in mouse iPSC generation are similar in human cells. Two million OS- (Figure 3C) or Oct4-infected (Figure 3D) HUVEC cells were seeded in 10-cm dishes and treated with combinations of compounds (including 0.5  $\mu$ M

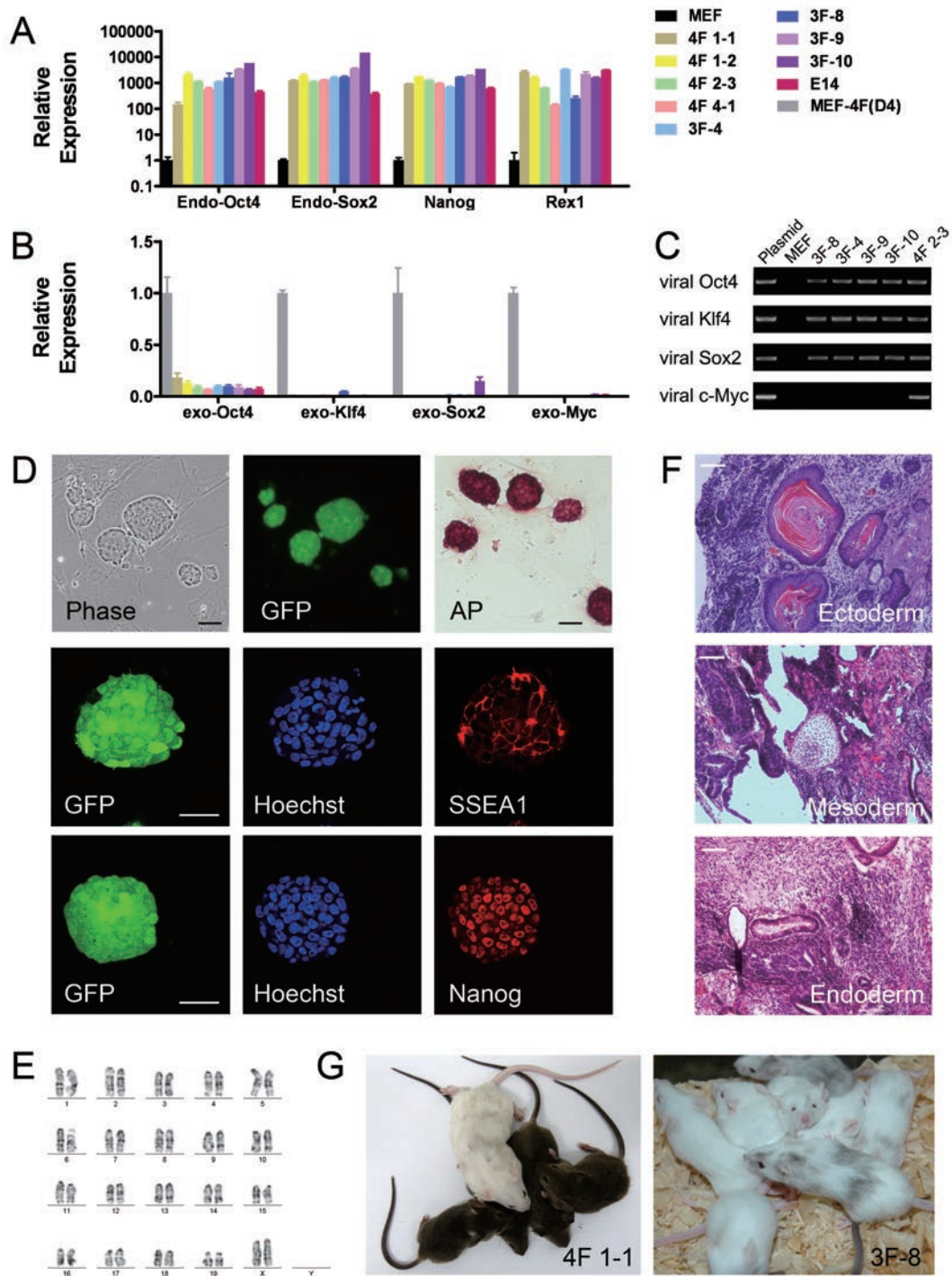
A83-01, 0.25 mM NaB, 5  $\mu$ M PS48, 3  $\mu$ M CHIR and 0.5  $\mu$ M PD0325901) in the presence of 5 mM LiCl or not. For OS-mediated reprogramming, Li was added from day 3-14; for Oct4-mediated reprogramming, Li was added from day 3-18. The other compounds were present at the full length of reprogramming until a week before colony counting and picking. Li doubled the reprogramming efficiency compared to the basal combinations of compounds in both OS- or Oct4-mediated hiPSC generation (Figure 3C and 3D). TRA-1-81<sup>+</sup> colonies were picked, reseeded and further characterized with immunofluorescent staining. Both OK-hiPSC (Figure 3E) and Oct4-hiPSC (Figure 3F) clones showed positive staining for pluripotency marker Nanog and SSEA4. These results indicate that Li not only promotes reprogramming with reduced factors, but also works well in human iPSC induction.

#### *Li's enhancement of iPSC generation is only partially mediated by GSK3 $\beta$ inhibition*

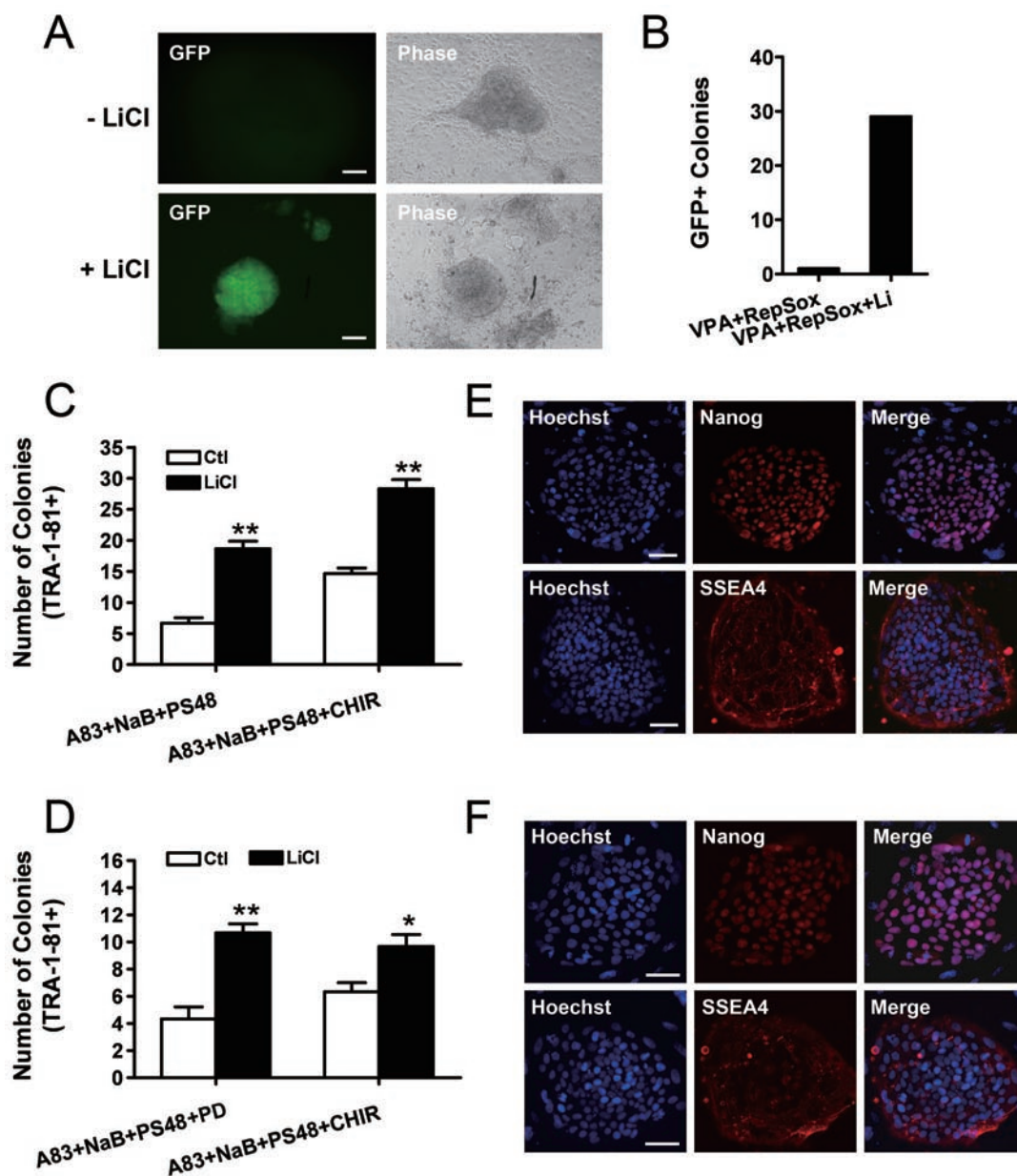
Li is well known to inhibit GSK3 $\beta$  [16]. GSK-3 $\beta$  inhibitors can maintain the undifferentiated state of human and mouse ES cells via activation of Wnt signaling [17]. A recent study indicated that dual inhibition (2i) of MAPK and GSK3 $\beta$  promotes the somatic cell reprogramming efficiency [18]. Therefore we compared Li's effect with three GSK3 $\beta$  inhibitors (BIO, CHIR99021 and SB415,286). We found that BIO and SB415,286 did not have any notable effect in our system at commonly applied concentrations, while CHIR99021 displayed marginal beneficial effect at 3  $\mu$ M (Figure 4A). As inhibition of GSK3 $\beta$  leads to stabilization and activation of  $\beta$ -catenin and TCF-dependent gene transcription [19], we used a TopFlash luciferase reporter system to compare the inhibitory effect of these chemicals on GSK3 $\beta$ . All four compounds inhibited GSK3 $\beta$ , CHIR99021 and BIO had slightly better inhibitory effect than Li (Figure 4B).

Another major target of Li is inositol monophosphatase (IMPase) [20]. Thus we measured the reprogramming efficiency of L-690,488, another IMPase inhibitor. The results indicated that L-690,488 did not have any notable effect at commonly applied concentrations (Figure 4A). Combination of CHIR99021 and L-690,488 slightly enhanced the reprogramming efficiency, but their effect was significantly lower than Li's (Figure 4C).

Previous study also showed that long-term Li treatment can suppress *P53* and *Bax* expression, but increase *Bcl-2* expression, and this plays important roles in neuroprotection [21]. Recent findings suggested that inhibition of *P53* and some of its downstream target gene expression can prevent cell senescence and significantly increase the reprogramming efficiency [22]. However, we



**Figure 2** Pluripotency of iPSC cells derived from MEFs with Li treatment. **(A, B)** qPCR analysis of pluripotency genes and exogenous transgenes in four 4F-iPSC clones and four 3F-iPSC clones generated with Li. mES cell line E14, MEF and MEF infected with 4F for 4 days (MEF-4F (D4)) were used as controls. **(C)** PCR analysis to confirm the absence of c-Myc integration in 3F-iPSC clones. **(D)** Top: morphology, GFP expression and AP staining in iPSC clone 4F 1-1. Middle and low: immunofluorescent staining of pluripotency gene SSEA-1 and Nanog in the same clone. Scale bar: 50  $\mu$ m. **(E)** Karyotype analysis of clone 4F 1-1. **(F)** HE-stained sections of teratomas formed with clone 4F 1-1. Typical structures of the three embryonic germ layers are shown: epidermis (ectoderm), muscle and cartilage (mesoderm) and epithelium (endoderm). Scale bar: 50  $\mu$ m. **(G)** Left: a chimeric mouse produced with iPSC clone 4F 1-1 and its agouti coat colored offspring. Right: chimeric mice produced with iPSC clone 3F-8.



**Figure 3** Lithium enhances the generation of mouse and human iPSCs with one or two factors. **(A)** 2F (OK)-infected MEFs were treated with 5 mM VPA and 1  $\mu$ M RepSox in combination with 5 mM LiCl or not. VPA and LiCl were added from day 3-13. Representative colonies were photographed at day 18. Scale bar: 50  $\mu$ m. **(B)** Number of GFP<sup>+</sup> colonies out of 150 000 starting OK-MEFs was counted at day 18. **(C, D)** 2 000 000 OS- **(C)** or Oct4-infected **(D)** HUVEC cells in 10-cm dishes were treated with combinations of compounds (including 0.5  $\mu$ M A83-01, 0.25 mM NaB, 5  $\mu$ M PS48, 3  $\mu$ M CHIR and 0.5  $\mu$ M PD0325901) in the presence of 5 mM LiCl or not. For OS-mediated reprogramming, LiCl were added from day 3-14; for Oct4-mediated reprogramming, LiCl were added from day 3-18. The other compounds were added from day 3-21 **(C)** or day 3-28 **(D)**. Colonies stained positive by Alexa Fluor 555 mouse anti-human TRA-1-81 antibody were counted at day 28 **(C)** and 35 **(D)**. Mean values  $\pm$  SEM of a representative experiment are shown,  $n = 3$ . \* $P < 0.05$ , \*\* $P < 0.01$ , versus control. **(E, F)** Immunofluorescent staining of pluripotency markers Nanog and SSEA4 in OK-hiPS **(E)** and Oct4-hiPS **(F)** clones. Scale bar: 50  $\mu$ m.

did not observe a significant change in the protein levels of P53 and BCL-2 with or without Li treatment during reprogramming (Supplementary information, Figure S2),

indicating that they are not involved in Li's beneficial effect in iPSC generation.

Taken together, these data suggest that Li's enhance-

ment of iPSC generation may only partially depend on GSK3 $\beta$  inhibition. Other reported targets of Li, such as IMPase, P53 and BCL-2, are not involved.

#### *Li increases the expression and transcriptional activity of Nanog*

Nanog plays a critical role in maintaining the pluripotent state of ES cells [23]. Endogenous expression of Nanog is considered to be essential for successful induction of iPSCs. Activation of Wnt/ $\beta$ -catenin pathway, or inhibition of GSK3 $\beta$  has been reported to increase Nanog expression [24] and Li has been reported to increase Nanog expression in some stem-like cells [10]. Using a luciferase reporter system (Addgene #16337) containing Nanog promoter region, we found that treatment with Li significantly activated (~2-folds) the Nanog promoter in HEK293T cells. CHIR99021 also significantly activated the Nanog promoter, though to a lesser extent (Figure 4D). During the 4F-mediated reprogramming, Li also induced ~2-fold increase in Nanog mRNA transcription (Figure 4E). Considering that Nanog can bind to its own promoter and form auto-regulation loop [25], we tested whether Li can enhance the transcriptional activity of Nanog. Interestingly, using a reporter system containing Nanog response element (binding site) [26], we found that Li significantly enhanced the expression of reporter in HEK293T cells co-expressed with Nanog. In contrast, CHIR99021 had no such effect (Figure 4F). Li enhanced reporter expression dependent on the presence of Nanog. In cells untransfected with Nanog, Li had no effect (Figure 4G). These results indicate that Li can increase the expression of Nanog gene by inhibiting GSK3 $\beta$ . But different from other GSK3 $\beta$  inhibitors, Li also enhances the transcriptional activity of Nanog.

#### *Li enhances iPSC generation by reducing LSD1 expression*

A recent study indicated that Li extended the life span of *C. elegans* by reducing the expression of LSD1 [27]. LSD1 shares significant sequence homology with FAD-dependent amine oxidases [28]. It has been found in a number of corepressor complexes, including CtBP, NRD, Co-REST and subsets of the HDAC complexes [28-30], and plays roles in transcriptional repression. A recent study revealed that LSD1 is a lysine-specific histone demethylase and specifically demethylates histone H3 lysine 4 (H3K4) [31]. Parnate, an amine oxidase inhibitor that also inhibits LSD1, was reported to promote reprogramming in combination with CHIR99021 [7]. Therefore we tested whether LSD1 was involved in Li-enhanced reprogramming. Prolonged Li treatment significantly reduced the LSD1 protein level in MEF

cells (Figure 4H and 4I). Meanwhile, the whole genome methylation level of H3K4 displayed a little but notable increase (Figure 4H and 4J). The effect of Li on LSD1 is not mediated by GSK3 $\beta$ , as CHIR99021 did not affect LSD1 level in MEFs (Supplementary information, Figure S3). Knocking down the expression of LSD1 with shRNA significantly enhanced the reprogramming efficiency of 4F-transduced MEF, though the enhancement only reached ~40% of Li's when the GFP<sup>+</sup> colonies were counted at day 10 and 16 (Figure 4K, 4L and 4M). Combination of Li and LSD1 shRNA displayed an additive effect on reprogramming efficiency in early stage (day 10, Figure 4L). Although in long term (day 16), the combination did not show better efficiency than Li alone (Figure 4M). This is probably due to the slow effect on LSD1 downregulation mediated by Li or shRNA alone. Combination of the two sped up this process and shortened the reprogramming time. And the combination did not show better overall efficiency at day 16 than Li alone, further confirming that LSD1 downregulation only contributed partly to Li's enhancement of reprogramming.

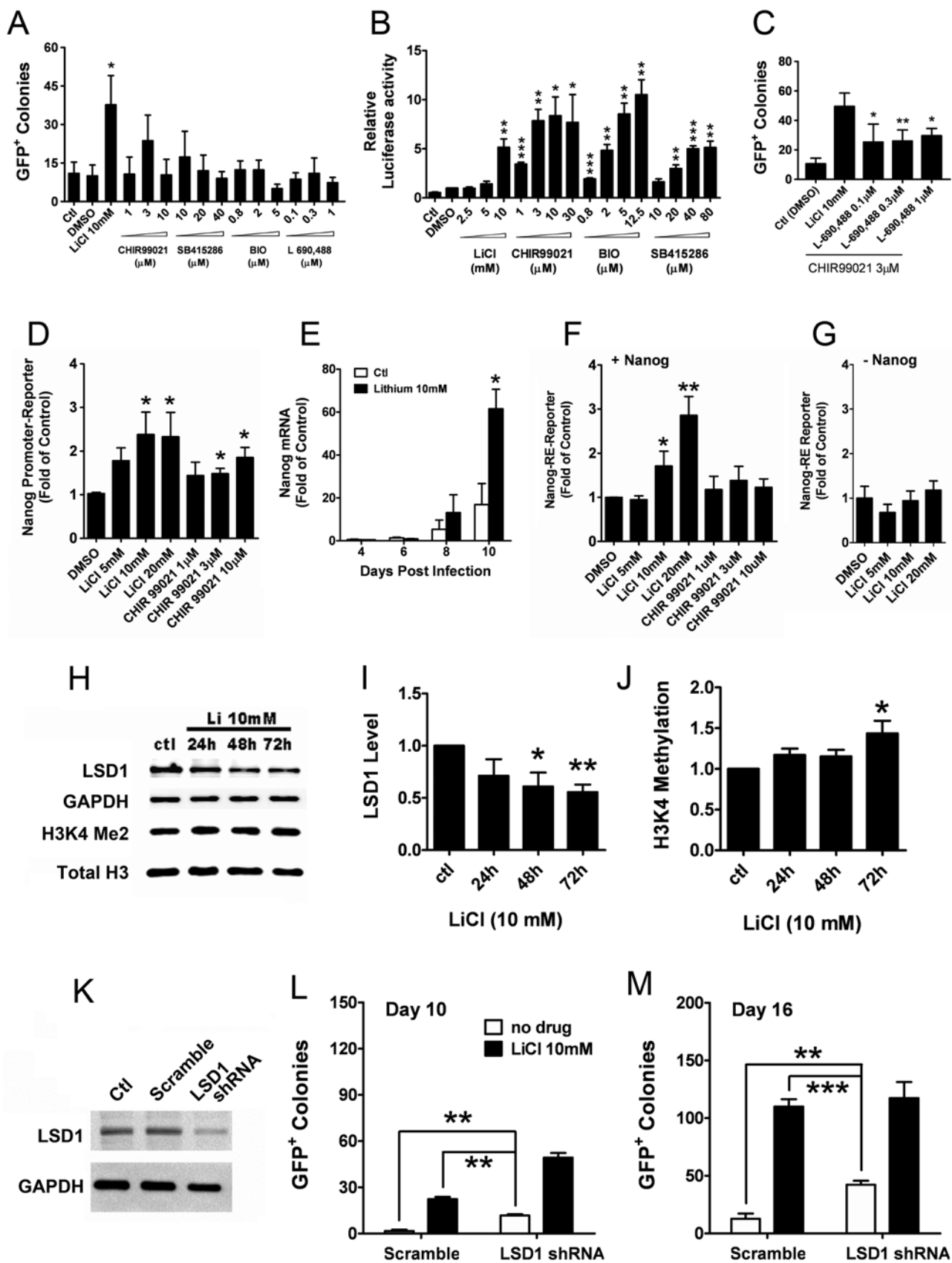
## Discussion

As one of the lightest elements in the periodic table, Li's therapeutic functions attracted many interests in the scientific fields. Li salts were started to be used therapeutically in the 19th century and have been used for bipolar disorder since the late 1940s. Li protects neurons from a variety of pro-apoptotic stimuli *in vitro* and *in vivo*. It also facilitates axonal remodeling and neurite outgrowth, which might help to restore neuronal functions (reviewed by [32]). Li is a potent stimulant of bone marrow stem cells and causes benign leukocytosis. It has been used clinically to restore leukocyte balance in various hematopoietic disorders. By increasing the count of neutrophils and eosinophils, Li inhibits T-cell production, and is sometimes used to modulate immune functions (reviewed by [9]).

In this study, we discovered that Li can enhance the reprogramming of somatic cells to iPSCs. By adding LiCl to the culture medium for a short period of time (day 3-8), we can obtain high-quality iPSCs with efficiency greater than 10% in both 4F- and 3F-induced reprogramming in MEF. Li also enhanced two factor (OS)- or one factor (Oct4)-mediated reprogramming in HUVEC, indicating that the mechanism of its action might be similar in both mouse and human system.

GSK3 $\beta$  is the best-studied target of Li. To our surprise, the reported reprogramming enhancer CHIR99021 [18], a synthetic GSK3 $\beta$  inhibitor, only displayed marginal ef-





fect in enhancing iPSC generation in our system. This is probably due to the difference in base media and supplements used in various studies, so the basal GSK3 $\beta$  activity might be different. Nevertheless, this indicates that GSK3 $\beta$  inhibition only contributes partly to Li's effect. Increased Nanog expression is one of the downstream effects of GSK3 $\beta$  inhibition [24]. Unlike other GSK3 $\beta$  inhibitors, Li not only increases the expression of Nanog, but also enhances the transcriptional activity of Nanog. Nanog can bind to its own promoter and form autoregulatory and feedforward circuitry [25], and Li may enhance such feedforward regulation. This may partially explain why Li works better than other GSK3 $\beta$  inhibitors. This also fits well with our data that Li is most effective when added from day 6-9, as the Nanog expression was a relative late event during reprogramming.

Li also seems to exert its effect by promoting epigenetic modifications. We found that prolonged Li treatment significantly reduces LSD1 protein level in MEF cells. Meanwhile, the whole genome methylation level of H3K4 displayed a little but notable increase, indicating activation of gene transcription. Knocking down LSD1 leads to significant enhancement of reprogramming efficiency, though the beneficial effect only reached ~40% of Li's. This provided first direct evidence that histone demethylases play critical roles in somatic cell reprogramming. How Li regulates LSD1 level is still not clear. It seems that Li's effect on LSD1 is not mediated by GSK3 $\beta$ , as CHIR99021 did not affect LSD1 level in MEFs. Whether Li targets LSD1 directly or via other pathways remains to be elucidated.

Other reported targets of Li, including IMPase, p53 and Bcl-2 were not involved in Li's enhancement of reprogramming. Li has also been proposed to modulate metabolic pathways and/or protein clearance systems, and alter cell organelles, mainly mitochondria and ER [33]. Mitochondria are the energy-regulating center of the cell and recently a small molecule regulating glycolysis has been reported to facilitate iPSC generation in combination with Oct4 and other chemicals [13]. Whether Li enhances reprogramming via metabolic pathways is still unclear. Our data also indicates a synergistic effect of Li and Vc and the mechanism also remains to be elucidated. It is also interesting that Li, Vc and VPA have all been reported to have anti-aging effect, and all three have such potent effect on reprogramming. Our findings not only provided a useful tool to help generate high-quality iPSC and dissect its underlying mechanisms, but also stimulate further research in the area of *in vivo* stem cell regulation and aging.

## Materials and Methods

### *Derivation of MEFs and cell culture*

OG2 mice, which carry the Rosa26-lacZ allele and a transgenic Oct4 promoter driving GFP expression [8, 34], were mated with 129 mice and MEF cells were isolated from e13.5 embryos heterozygous for the *Oct4*-GFP transgenic allele. Gonads and internal organs were removed before processing for isolation of MEF cells. MEFs were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamax, 0.1 mM nonessential amino acids (NEAA), 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. Isolated MEF cells in early passages (up to passage 3) were used for

**Figure 4** Involvement of GSK3 $\beta$ , Nanog and LSD1 in Li's effect of promoting reprogram. **(A)** OKSM-infected MEFs were treated with GSK3 $\beta$  inhibitors (CHIR99021, SB415286 and BIO) and IMPase inhibitor L690,488, and GFP<sup>+</sup> colonies were counted. Mean values  $\pm$  SEM of a representative experiment measured in triplicate are shown. \* $P$  < 0.05, versus control. **(B)** Wnt pathway activation by GSK3 $\beta$  inhibitors. 293T cells were transfected with 8 $\times$  TopFlash reporter and treated with various GSK3 $\beta$  inhibitors. Mean values  $\pm$  SEM of three independent experiments are shown. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001. **(C)** OKSM-infected MEFs were treated with LiCl or the combination of GSK3 $\beta$  inhibitor (CHIR99021) and IMPase inhibitor (L690,488), and GFP<sup>+</sup> colonies were counted. Mean values  $\pm$  SEM of a representative experiment measured in triplicate are shown. \* $P$  < 0.05, \*\* $P$  < 0.01, versus LiCl treatment. **(D)** Nanog reporter activation by GSK3 $\beta$  inhibitors. 293T cells were transfected with a reporter construct containing Nanog promoter region (p5N nanog reporter) and treated with LiCl and CHIR99021. Mean values  $\pm$  SEM of three independent experiments are shown, \* $P$  < 0.05, \*\* $P$  < 0.01, versus control. **(E)** qPCR analysis of Nanog expression during iPSC generation in OKSM-infected MEF cells treated with or without LiCl. Expression levels were normalized using GADPH and mean values  $\pm$  SEM of three independent experiments are shown. \* $P$  < 0.05, versus control. **(F,G)** Enhancement of Nanog transcriptional activity by Li. 293T cells were transfected with a reporter construct containing Nanog response element (Nanog5P reporter) in the presence of Nanog or not, then treated with LiCl or CHIR99021. Mean values  $\pm$  SEM of three independent experiments are shown, \* $P$  < 0.05, \*\* $P$  < 0.01, versus control. **(H)** Representative western blot to detect the expression of LSD1 and methylation of H3K4 after LiCl treatment in MEFs. **(I, J)** Statistical analysis of LSD1 level and H3K4 methylation with western blot. Mean values  $\pm$  SEM of three independent experiments are shown, \* $P$  < 0.05, \*\* $P$  < 0.01, versus control. **(K)** Western blot analysis to determine the efficiency of LSD1 shRNA. Lysates were extracted from MEF cells 2 days post infection. **(L, M)** MEFs were infected with OKSM together with scramble or LSD1 shRNA. LiCl was added to the culture from day 3-8. GFP<sup>+</sup> colonies were counted at day 10 and 16. Mean values  $\pm$  SEM of a representative experiment measured in triplicate are shown. \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, versus control.

further experiments.

### Mouse iPSC generation

Retrovirus were produced by transfection of plat-E cells with pMXs retroviral vectors containing the coding sequences of mouse Oct4, Sox2, Klf4 and c-Myc (obtained from Addgene). MEFs were seeded at a density of 150 000 cells per well in six-well plate 18 h before infection. Virus containing supernatants, supplemented with 4 µg/ml polybrene, were added onto the plates of MEF cell cultures and spined at 2 500 rpm for 90 min to ensure their infection. Medium was changed immediately after virus transduction and this day is counted as “Day 0”. Two days post virus infection, MEFs were digested into single cells and reseeded at a density of 5 000 cells per well on 96-well plates or 7 500 cells per well on 24-well plates pre-seeded with irradiated MEF feeders, supplemented with mES medium (DMEM supplemented with 15% FBS, 2 mM L-glutamax, 0.1 mM NEAA, 0.1 mM β-mercaptoethanol 1 000 U/ml LIF, 100 units/ml penicillin and 100 µg/ml streptomycin). At day 6, culture medium was replaced with KSR medium (knockout-DMEM supplemented with 15% knockout serum replacement, 2 mM L-glutamax, 0.1 mM nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol, 1 000 U/ml LIF, 100 units/ml penicillin and 100 µg/ml streptomycin). Chemicals were added from day 3 with various durations.

GFP<sup>+</sup> colonies were counted using an Olympus IX51 inverted fluorescent microscope from the day 8 post infection. Images of representative wells were taken and stitched in Photoshop. GFP<sup>+</sup> colonies were also trypsinized and then analyzed using a FACS Calibur (BD). GFP<sup>+</sup> cells were gated with a control signal from the PE channel and a minimum of 10 000 events were recorded.

### Human iPSC generation

HUVECs (Millipore) were maintained in EndoGRO-VEGF complete medium (HCM, CHEMICON). The lentivirus supernatants were produced and harvested as previously described [2]. The plasmids used for lentivirus production include pSin-EF2-Puro-hOCT4 and pSin2-EF2-Puro-hSOX2. 200 000 transduced HUVECs were seeded on gelatin coated 100-mm dish cultured in HCM and transduced twice (4-6 h each transduction) with freshly produced lentivirus supernatants, supplemented with 4 µg/ml polybrene. Medium was changed immediately after virus transduction and the day of first infection is counted as “day 0”. For Oct4/Sox2-induced reprogramming, LiCl (5 mM) was added to the culture from day 3 to 14, and a combination of compounds including PS48 (5 µM), NaB (0.25 mM), A-83-01 (0.5 µM) and CHIR99021 (3 µM) were supplemented to the culture from day 3 to 21. For Oct4 induced reprogramming, LiCl (5 mM) was added to the culture from day 3 to 18 and the compound combinations were supplemented from day 3 to 28. The iPSC colonies stained positive by Alexa Fluor 555 mouse anti-human TRA-1-81 antibody were counted and picked up for expansion on feeder cells in hES medium and cultured routinely.

### AP and immunofluorescent staining

For AP staining, iPS cells were fixed with 4% paraformaldehyde (PFA) in PBS for 45 s, rinsed once with PBS and detection was performed using a leukocyte AP kit (Sigma, catalog No 85L3R) according to the manufacturer’s protocol. For immunofluorescent staining, cells were fixed with 4% PFA and incubated

with primary antibodies against mSSEA-1 (Santa Cruz, sc-21702) and mNanog (Millipore, AB5731), followed by the appropriate secondary antibodies conjugated to Alexa Fluor 555 (Invitrogen). Nuclei were counterstained with Hoechst 33342 (Sigma). Images were taken with an Olympus IX51 inverted fluorescent microscope or an Olympus FV10i confocal microscope.

### Real-time PCR

Total mRNA was isolated using TRIzol (Invitrogen) and 2 µg RNA were used to synthesize cDNA using PrimeScript<sup>TM</sup> RT reagent kit (Takara, DRR037A) according to the manufacturer’s protocol. Real-time PCR was performed using JumpStart<sup>TM</sup> TaqReady Mix<sup>TM</sup> (Sigma, D7440) with Eva Green (Biotium) and analyzed with a Stratagene Mx 3000P thermal cycler. For semi-quantitative PCR analysis, the cDNA solution was amplified for 30 cycles at an optimal annealing temperature. Primers sequences are supplied in Supplementary information, Table S1.

### Western blot

Cells were lysed, sonicated and boiled at 95-100 °C for 5 min in sample buffer (50 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 1% β-mercaptoethanol, 0.01% bromophenyl blue (pH 6.8)). Cell lysates were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were first incubated with blocking buffer (TBS with 0.05% Tween 20, 10% nonfat milk) for 1 h at room temperature and then incubated overnight at 4 °C in buffer containing rabbit anti-GAPDH (1:10 000; CST), mouse anti-p53 (1:1 000; CST), rabbit anti-Bcl-2 (1:1 000; Bioworld), rabbit anti-LSD1 (1:1 000; Abcam) and rabbit anti-phosph-GSK3β (1:2 000 dilution; CST). The membranes were washed thrice and incubated with goat anti-rabbit IgG HRP (1:10 000) or goat anti-mouse IgG HRP (1:10 000) for 1 h. After washing, immunostaining was visualized using Amersham ECL Plus Western Blotting detection reagents (GE Healthcare).

### Teratoma formation, chimera production and germline transmission

About  $1 \times 10^6$  iPS cells were suspended in 200 µl mES medium and injected into NOD-SCID mice to form teratomas. Four weeks after injection, teratomas were harvested, fixed overnight with 4% PFA, embedded in paraffin, sectioned, HE stained and analyzed. For production of chimeric mice, zygotes were isolated from superovulated female ICR mice and iPS cells were injected into the resulting blastocysts. Chimeras were produced by implantation of injected blastocysts into the pseudopregnant ICR mice. The chimeras were crossed with ICR mice to confirm the germline transmission.

### Reporter assay

For 8× TOP Flash reporter activity, 1.2 µg of 8× TOP Flash reporter construct and 0.1 µg Renilla luciferase construct (pCMV, Promega) were co-transfected into 15 million HEK293T cells using electroporation. Chemicals were added 18 h after transfection; firefly and renilla luciferase activities were measured 6 h after drug treatment with the Dual-Glo Luciferase Assay System (Promega) using a EnVision Multilabel Reader (Perkin Elmer). To test Nanog transcriptional activity, 3 µg p5N nanog reporter construct [26], 1.6 µg HA-tagged mouse Nanog and 0.1 µg Renilla luciferase construct were co-transfected. Chemicals were added 18 h after

transfection; firefly and renilla luciferase activities were measured 24 h after with the Dual-Glo Luciferase Assay System. For Nanog reporter activity, 3 µg Nanog5P reporter (Addgene) and 0.1 µg Renilla luciferase construct were co-transfected. Chemicals were added 18 h later and dual-luciferase activities were measured 24 h later.

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(**Supplementary information** is linked to the online version of

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