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ORIGINAL ARTICLE

Stress-mediated p38 activation promotes somatic cell reprogramming

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Environmental stress-mediated adaptation plays essential roles in the evolution of life. Cellular adaptation mechanisms usually involve the regulation of chromatin structure, transcription, mRNA stability and translation, which eventually lead to efficient changes in gene expression. Global epigenetic change is also involved in the reprogramming of somatic cells into induced pluripotent stem (iPS) cells by defined factors. Here we report that environmental stress such as hyperosmosis not only facilitates four factor-mediated reprogramming, but also enhances two or one factor-induced iPS cell generation. Hyperosmosis-induced p38 activation plays a critical role in this process. Constitutive active p38 mimics the positive effect of hyperosmosis, while dominant negative p38 and p38 inhibitor block the effect of hyperosmosis. Further study indicates stress-mediated p38 activation may promote reprogramming by reducing the global DNA methylation level and enhancing the expression of pluripotency genes. Our results demonstrate how simple environmental stress like hyperosmosis helps to alter the fate of cells via intracellular signaling and epigenetic modulation.
Keywords: induced pluripotent stem cells; iPS; hyperosmosis; NaCl; stress; p38; reprogramming
Cell Research (2013) **23**:131-141. doi:10.1038/cr.2012.143; published online 9 October 2012

Introduction

Environmental stress has long been associated with numerous human diseases. Nevertheless, not all stress is harmful. Instead, environmental stress below the mutation threshold might play an essential role in the adaptation and evolution of life [1]. Classical responses of animals to stress, such as the 'fight-or-flight response' or 'general adaptation syndrome', are controlled by hormones at the organismal level [2]. At the cellular level, the adaptation mechanisms usually involve efficient changes in the gene expression programs mediated by intracellular signaling networks. Regulation of chromatin structure, transcription, mRNA stability and translation are some of the critical steps for cells to achieve generic and specific responses to different stresses [3].

Somatic cells can be reprogrammed into induced

pluripotent stem (iPS) cells by ectopic expression of defined factors [4]. The exact underlying mechanisms remain unclear, but a global epigenetic change is believed to be involved in iPS induction [5]. To avoid the use of oncogenes and incorporation of viral DNA sequences, and to make iPS cells more amenable for therapeutic application, many efforts have been taken to modify the original protocol by using reduced number of factors [6], non-integrating gene delivery approaches [7], or cell permeable proteins to trigger the reprogramming [8]. A number of small molecules, especially those affecting epigenetic modulations, such as VPA, butyrate, BIX-01294 and 5-Aza-2′-deoxycytidine (5-Aza), have also been reported to facilitate iPS cell induction [9].

To identify better small molecule enhancers of reprogramming, we developed a screening system with 96-well plates. Unexpectedly, we found that iPS cells appeared earlier and the induction efficiency was higher in the corner wells than the center ones. After ruling out the possible effect of pH and nutritional changes, we discovered that it was the increased osmotic pressure in the corner wells that facilitated iPS cell generation. Hyperosmosis not only facilitated four factor-mediated repro-

gramming, but also enhanced two or one factor-induced iPS cell generation in combination with other chemicals. Hyperosmosis has been reported to activate many stress-related signaling pathways, including the MAPK pathways [10]. Our study indicated that hyperosmosis-induced p38 activation played a critical role in facilitating reprogramming. The constitutive active p38 mimicked the positive effect of hyperosmosis, while dominant negative p38 and p38 inhibitor PD169316 blocked the effect of hyperosmosis. Further study indicates stress-mediated p38 activation might promote reprogramming by reducing the global DNA methylation level and enhancing the expression of pluripotency genes. We thus demonstrate how simple environmental stress like hyperosmosis could help to alter the fate of cells via intracellular signaling and epigenetic modulation.

Results

Hyperosmosis promotes four factor-mediated reprogramming in mouse embryonic fibroblasts (MEFs)

We established a 96-well-plate-based chemical screening system for four-factor (4F)-induced reprogramming in OG2 MEFs which carry a transgenic *Oct4* promoter driven-GFP reporter [11] (Supplementary information, Figure S1A). We unexpectedly observed that the reprogramming efficiency at the corner wells were higher than the center ones. Corner or edge effects are generally associated with increased osmotic pressure and pH. By using NaCl and $NaHCO_3$ to mimic the osmotic and pH changes, we found only the addition of NaCl (50-100 mM) significantly increased the number of GFP$^+$ colonies. Change of pH or the percent of serum did not affect iPS cell generation (Supplementary information, Figure S1B). More careful analyses indicated NaCl supplementation not only increased the number of GFP$^+$ colonies, but also shortened the reprogramming process. At day 8, more than 10 GFP$^+$ colonies could be observed in NaCl-added wells (5 000 MEFs/well), while the control wells had almost none (Figure 1A). The non-ionic osmotic pressure regulator, sucrose (200 mM) [12], also increased the reprogramming efficiency by ~10-fold in 4F-transduced MEFs (Figure 1B). At day 14, FACS data revealed a remarkable enhancement of reprogramming efficiency with > 20% cells being GFP$^+$ (Figure 1C).

Hyperosmosis is effective when implemented at the early stage of reprogramming and the effect is additive to other chemicals

To clarify whether hyperosmosis facilitates the reprogramming process or promotes the self-renewal of iPS cells after reprogramming, we treated the 4F-transduced MEF cells with 100 mM NaCl for 72 h starting on day 3, 6, or 9. Starting NaCl treatment on day 9 had no beneficial effect, instead it slightly reduced the number of GFP$^+$ colonies. In contrast, there was a statistically significant 8- and 6-fold increase in the number of GFP$^+$ colonies in the cultures treated with NaCl starting on day 3 and 6, respectively (Figure 1D and Supplementary information, Figure S1C). We also treated the 4F-transduced MEF cells with 50 mM or 100 mM NaCl for various durations starting from day 3. The early stage of reprogramming (day 3-8) benefited most from the hyperosmotic stress, since prolonged elevation of osmotic pressure did not further increase the reprogramming efficiency (Figure 1E). In fact, prolonged hyperosmotic stress (treatment beyond day 10) caused reduction in colony size and eventual reduction in colony number, indicating a cytotoxic effect. So the treatment duration for most of the later experiments was day 3-8. On fully reprogrammed iPS clones, hyperosmosis reduced the GFP$^+$ area and total GFP intensity, indicating the condition was harmful to iPS cells (Supplementary information, Figure S1D and S1E). MTT assay also revealed reduced viability in iPS cells when cultured in hyperosmotic condition (Supplementary information, Figure S1F). These data suggest that hyperosmosis promotes the generation of iPS colonies by facilitating the reprogramming process rather than enhancing the proliferation or self-renewal of iPS cells.

Next we tested NaCl in combination with two reported reprogramming enhancers, VPA and LiCl [13-14]. The combination of NaCl and VPA at low doses displayed a synergistic effect in enhancing reprogramming, while the combination of high doses did not even show additive effect. Similar to NaCl, VPA also reduced the GFP$^+$ area, total GFP intensity and cell viability in fully reprogrammed iPS cells (Supplementary information, Figure S2C-S2E). The combination of VPA and NaCl at high doses displayed higher toxicity towards iPS cells, which might compromise their effects in enhancing reprogramming. Similar synergistic effect between low dose of NaCl and low dose of LiCl was also observed (Supplementary information, Figure S2B).

Hyperosmosis facilitates the generation of mouse iPS cells with two or one factors and the iPS cells generated in such conditions are pluripotent

Hyperosmosis also facilitated reprogramming mediated by reduced number of factors and chemicals (Figure 1F). In OK, CHIR99021 and parnate-mediated reprogramming, NaCl (50 mM) led to a 5-fold increase in the number of GFP$^+$ colonies at day 14. In OS, CHIR99021, Repsox and parnate-mediated reprogramming, hyperos-
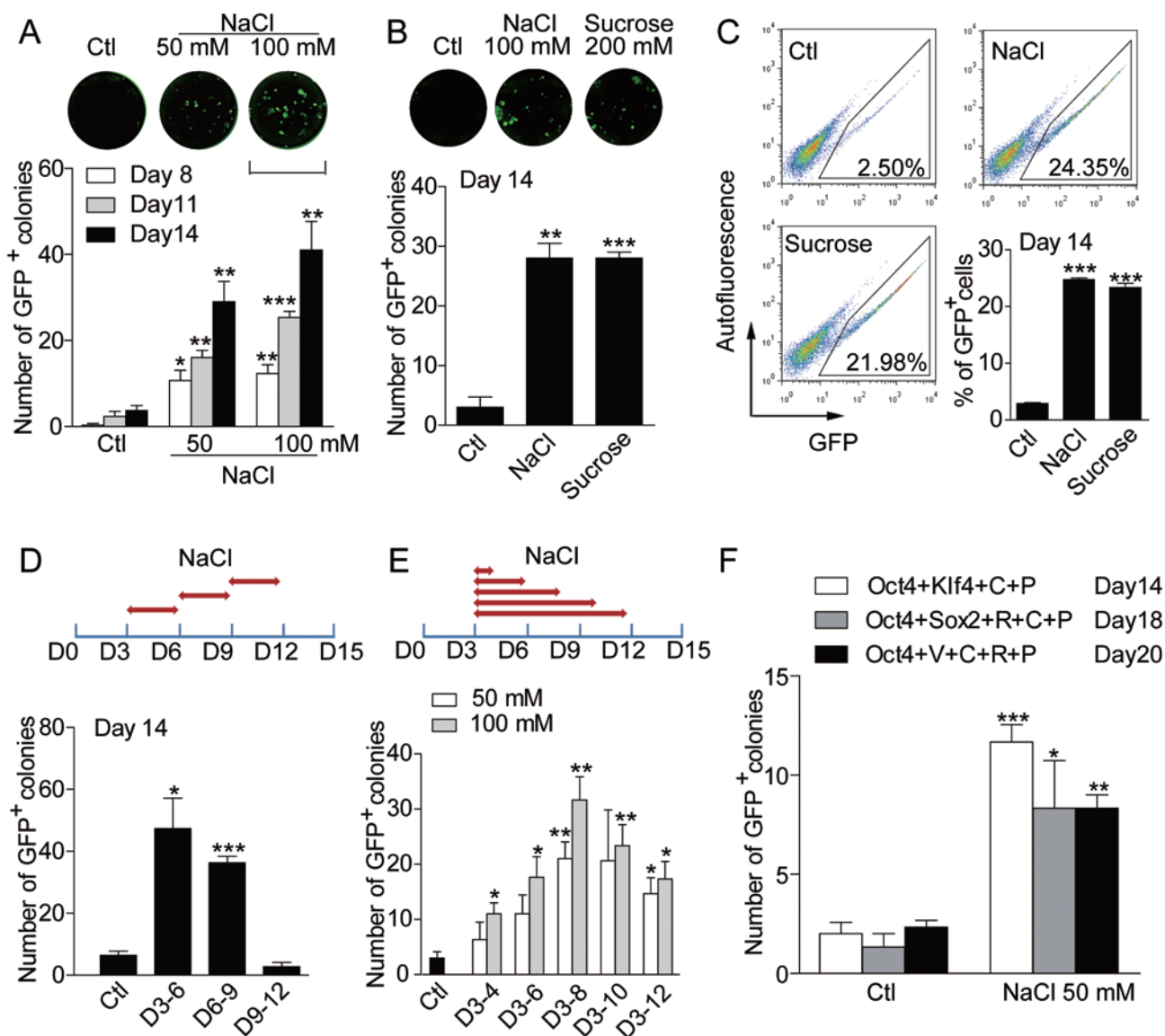
Figure 1 Hyperosmosis promotes iPS cell generation from MEFs. **(A)** Representative image and statistical data of the dose and time effect of NaCl on iPS (GFP$^+$) colony generation from 5 000 4F-infected MEFs. Scale bar, 7 mm. **(B)** The effect of NaCl (100 mM) or sucrose (200 mM) in iPS cell induction in 4F-infected MEFs. **(C)** Representative FACS plots and statistical data of GFP$^+$ cell percentage at day 14 from 4F-infected MEFs treated with NaCl (100 mM) or sucrose (200 mM). **(D)** NaCl (100 mM) was added for 3 days starting from day 3, 6 and 9 in 4F-infected MEFs and GFP$^+$ clonies were counted at day 14. Mean ± SEM of a representative experiment, n = 3. **(E)** 4F-infected MEFs were treated with NaCl for various durations starting from day 3, and GFP$^+$ colonies were counted at day 14. Mean ± SEM of three independent experiments. **(F)** MEFs infected with 2F or 1F were treated with a combination of small molecules including 0.5 mM VPA (V), 3 μM CHIR99021 (C), 1 μM RepSox (R) and 2 μM parnate (P) supplemented with 50 mM NaCl or not. Number of GFP$^+$ colonies was counted. Mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus control.

motic condition further improved the efficiency by 4-fold at day 18. And the effect of the chemical combination (VPA, CHIR99021, parnate and Repsox) that successfully facilitated the one factor (Oct4)-induced reprogramming was further enhanced by 3-fold in the hyperosmotic condition when examined at day 20 (Figure 1F).

PCR of genomic DNA of 2F (OK or OS)- or 1F(O)-iPS clones confirmed the integration of the indicated but not the other retroviral factors (Supplementary information, Figure S3A and S3B). Real-time PCR analysis

confirmed the reactivation of the endogenous *Oct4*, *Sox2*, *Nanog* and *Rex1* and the silencing of viral genes in these iPS clones (Supplementary information, Figure S3C and S3D). These iPS cells maintain GFP expression and ES-like morphology, and express typical pluripotency markers, such as alkaline phosphatase, SSEA1 and Nanog (Figure 2A). Bisulfite genomic sequencing analyses of the *Oct4* and *Nanog* promoters revealed that both were demethylated in iPS cells, similar to mES cells and in contrast to the parental MEFs (Figure 2B). Subcutaneous injection of the iPS cells into NOD-SCID mice led to teratoma formation, with tissues derived from all three germ layers (Figure 2C). We obtained adult chimaeras from all three clones (OK-1, OS-1 and O-1) as determined by coat color (Figure 2D and Supplementary information, Table S3). One of the male chimaeras from clone OK-1 was crossed with ICR females and 7 of the total 47 F1 mice showed agouti coat color (Figure 2E and Supplementary information, Table S3), confirming germline transmission.

Hyperosmosis promotes iPS cell generation by activating the p38 pathway

Hyperosmosis was reported to activate many stress-related signaling pathways [10]. We found hyperosmosis significantly activated JNK and p38, two major regulators of stress responses, and to a lesser extent, the ERK pathway (Figure 3A and 3B). Blocking JNK and ERK pathways with SP600125 and PD98059 further enhanced the positive effect of hyperosmosis on reprogramming (Figure 3C). The result agrees with previous reports that the activation of ERK and JNK pathways was detrimental to iPS cell induction [15]. Interestingly, blocking hyperosmosis induced-p38 activation with PD169316 totally abolished its promoting effect on reprogramming (Figure 3C). Anisomycin and TNF-α, two well known activators of p38 and its downstream target MAPKAPK2 (MK2) (Supplementary information, Figure S4A, S4B and S4D), also enhanced iPS cell generation and such enhancement was blocked by PD169316 (Figure 3D). Sucrose also activated the p38 pathway in MEF cells (Supplementary information, Figure S4C). In contrast, VPA was believed to enhance reprogramming via HDAC inhibition. It did not induce p38 activation and its effect was not blocked by PD169316 (Supplementary information, Figure S4E and S4F).

To confirm the role of p38 in reprogramming, we constructed the constitutively active (CA)-p38, which contains D176A and F327S mutations [16], and the dominant negative (DN)-p38 with T180A and Y182F mutations [17]. Western blot analysis confirmed the expression and correct function of these constructs (Sup-

plementary information, Figure S5). When expressed in MEFs, only the CA-p38 promoted 4F-driven iPS cell generation in standard iso-osmotic medium. WT-p38 and DN-p38 had no effect. However, in hyperosmotic medium, DN-p38 totally blocked hyperosmosis-enhanced reprogramming (Figure 3E). Similar to NaCl treatment, the CA-p38 was only beneficial at the reprogramming stage. If CA-p38 was blocked with PD169316 at early reprogramming stage (before day 9), no enhancement was observed (Figure 3F). But if PD169316 was added after day 15, prolonged activation of p38 also caused reduction in iPS colonies (Figure 3F). The time courses of NaCl and CA-p38 on reprogramming were slightly different, which was probably due to the variable degrees of p38 activation.

Hyperosmotic stress-mediated p38 activation may promote reprogramming by reducing the global DNA methylation level

Endogenous expression of *Nanog*, *Oct4* and *Sox2* is essential for successful reprogramming of somatic cells. During the 4F-mediated reprogramming, hyperosmotic stress induced a significant increase in *Nanog*, *Oct4* and *Sox2* mRNA transcription (Figure 4A). By using luciferase reporters driven by the promoters of *Nanog*, *Oct4* or *Sox2*, we found hyperosmosis significantly activated all these promoters without the presence of Yamanaka factors (Figure 4B). Interestingly, p38 inhibitor PD169316 (10 μM) only blocked the activation of *Nanog* and *Sox2* reporters, but not the *Oct4* reporter (Figure 4B), indicating the involvement of other pathways in the activation of *Oct4* transcription by hyperosmosis. DNA methylation levels at the loci of *Oct4* and *Nanog* promoters after NaCl treatment were determined by bisulfite sequencing. As shown in Figure 4C, 4D and Supplementary information, Figure S6, more than 60% of the CpGs in the promoter regions of *Oct4* and *Nanog* were methylated in MEFs. In contrast, ES cells had almost no methylation in the promoter regions of these genes. NaCl treatment significantly reduced the level of methylation in both *Oct4* and *Nanog* promoters, supporting that hyperosmotic conditions can facilitate the activation of these pluripotency genes.

Hyperosmotic stress has been reported to activate many genes critical for cell differentiation, including Keratin K1 and K10 for epidermal differentiation, and MEF2 for skeletal muscle differentiation [18-19]. P38 activation has also been found to induce cell differentiation [20]. The fact that hyperosmosis and p38 activation facilitate both differentiation and reprogramming led us to speculate that stress might affect gene transcription at a global level. Using dot blot analysis, we found
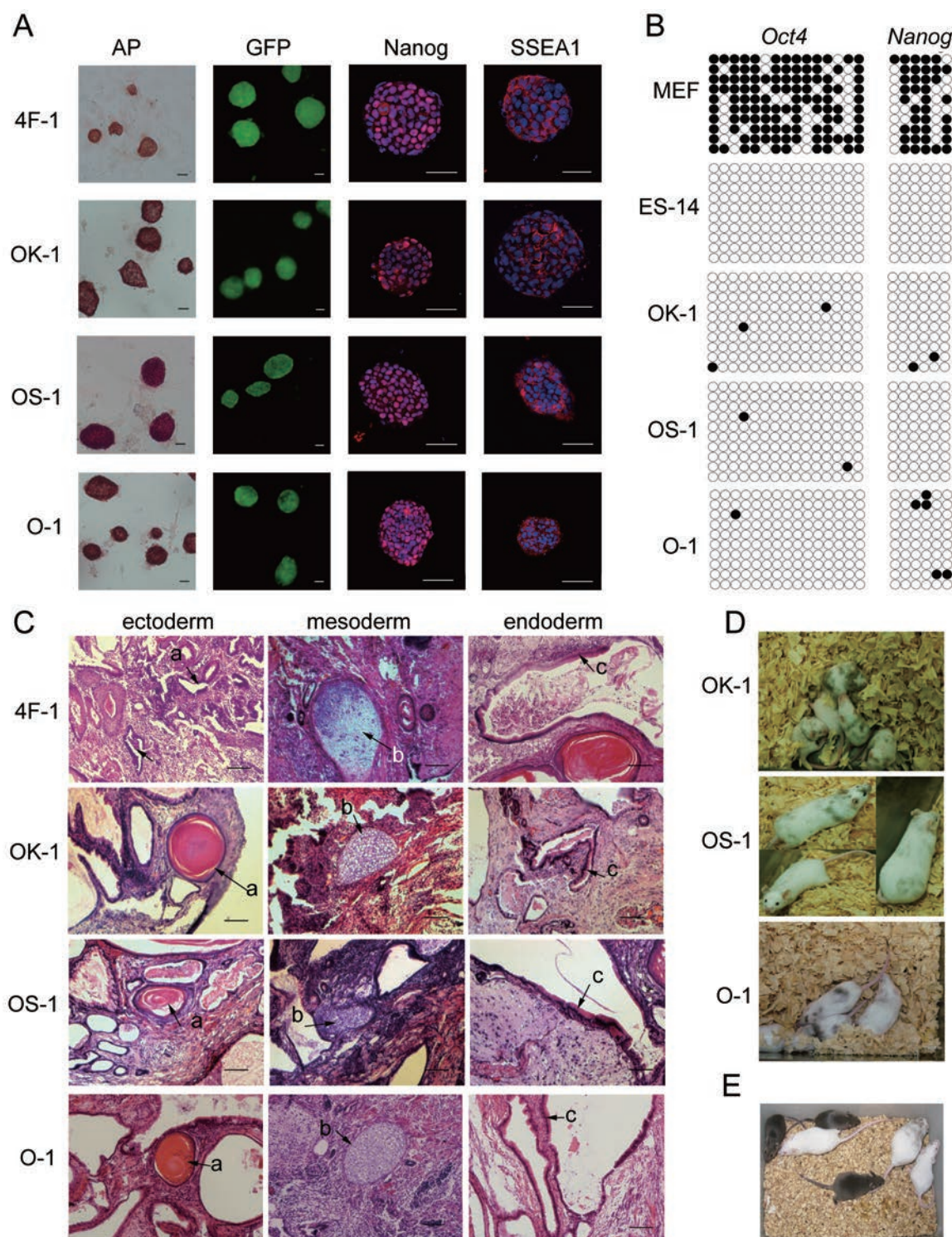
Figure 2 Pluripotency of iPS cells generated in hyperosmotic conditions. **(A)** Alkaline phosphatase staining, GFP expression and immunofluorescent staining of pluripotency marker SSEA-1 and Nanog in 4F-, 2F (OK or OS)- and 1F (O)-iPS clones. Scale bar, 50 μm. **(B)** DNA methylation analysis of the *Oct4* and *Nanog* promoters by bisulfite genomic sequencing in ES-14 and iPS clones. Open and closed circles indicate unmethylated and methylated CpGs, respectively. **(C)** Hematoxylin and eosin-stained sections of teratomas formed with iPS clones. Typical structures of the three embryonic germ layers were pointed out by arrows: a, epidermis (ectoderm), b, cartilage (mesoderm) and c, glandular epithelium (endoderm). Scale bar, 50 μm. **(D)** Chimeric mice produced with 2F- and 1F-iPS clones. **(E)** A chimeric mouse generated with iPS clone OK-1 and its agouti-coat-colored offsprings.
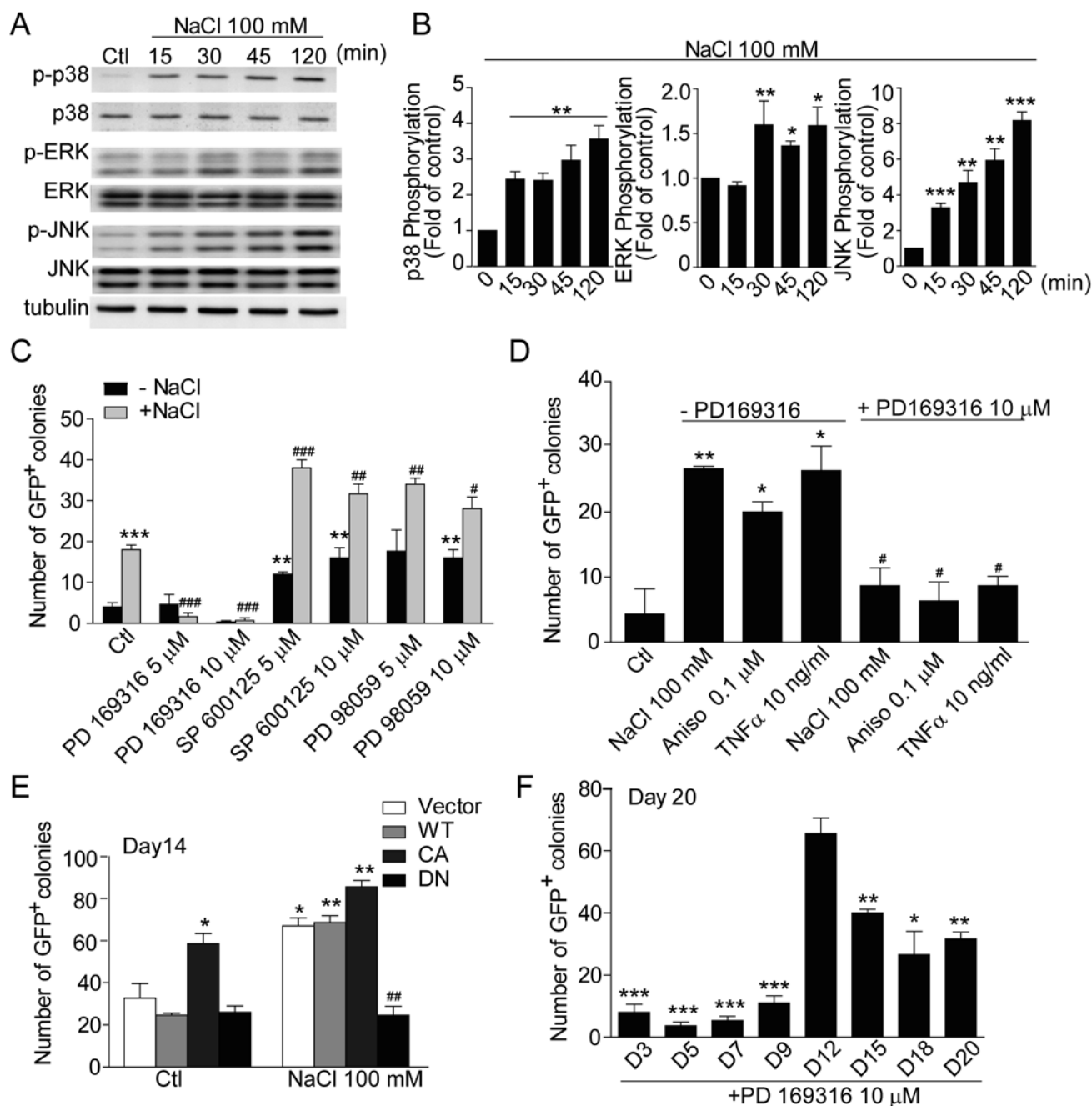
Figure 3 Hyperosmosis promotes reprogramming via p38 activation. **(A)** Representative western blot of p38, JNK and ERK phosphorylation after NaCl treatment in MEFs. **(B)** Statistical analysis of A. Mean ± SEM of three independent experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(C)** The effect of p38 inhibitor PD169316, JNK inhibitor SP600125 and ERK inhibitor PD98059 on hyperosmosis-enhanced reprogramming in 4F-infected MEFs. Mean ± SEM of a representative experiment, n = 3, ** $P < 0.01$, *** $P < 0.001$, versus blank control (first black bar). #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, versus NaCl control (first gray bar). **(D)** The effect of anisomycin and TNFα on reprogramming in the presence of PD196316 or not. Mean ± SEM of a representative experiment, n = 3. * $P < 0.05$, ** $P < 0.01$, versus control; # $P < 0.05$ versus no PD169316 treatment groups. **(E)** MEFs were infected with 4F and WT-, CA- or DN-p38, and cultured in medium supplemented with NaCl or not, and GFP+ colonies were counted at day 14. Mean ± SEM of a representative experiment, n = 3. * $P < 0.05$, ** $P < 0.01$, versus vector control without NaCl. ##$P < 0.01$ versus vector control with NaCl. **(F)** MEFs infected with 4F and CA-p38 were treated with PD169316 from indicated days. GFP+ colonies were counted at day 20. Mean ± SEM of a representative experiment, n = 3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus D12.
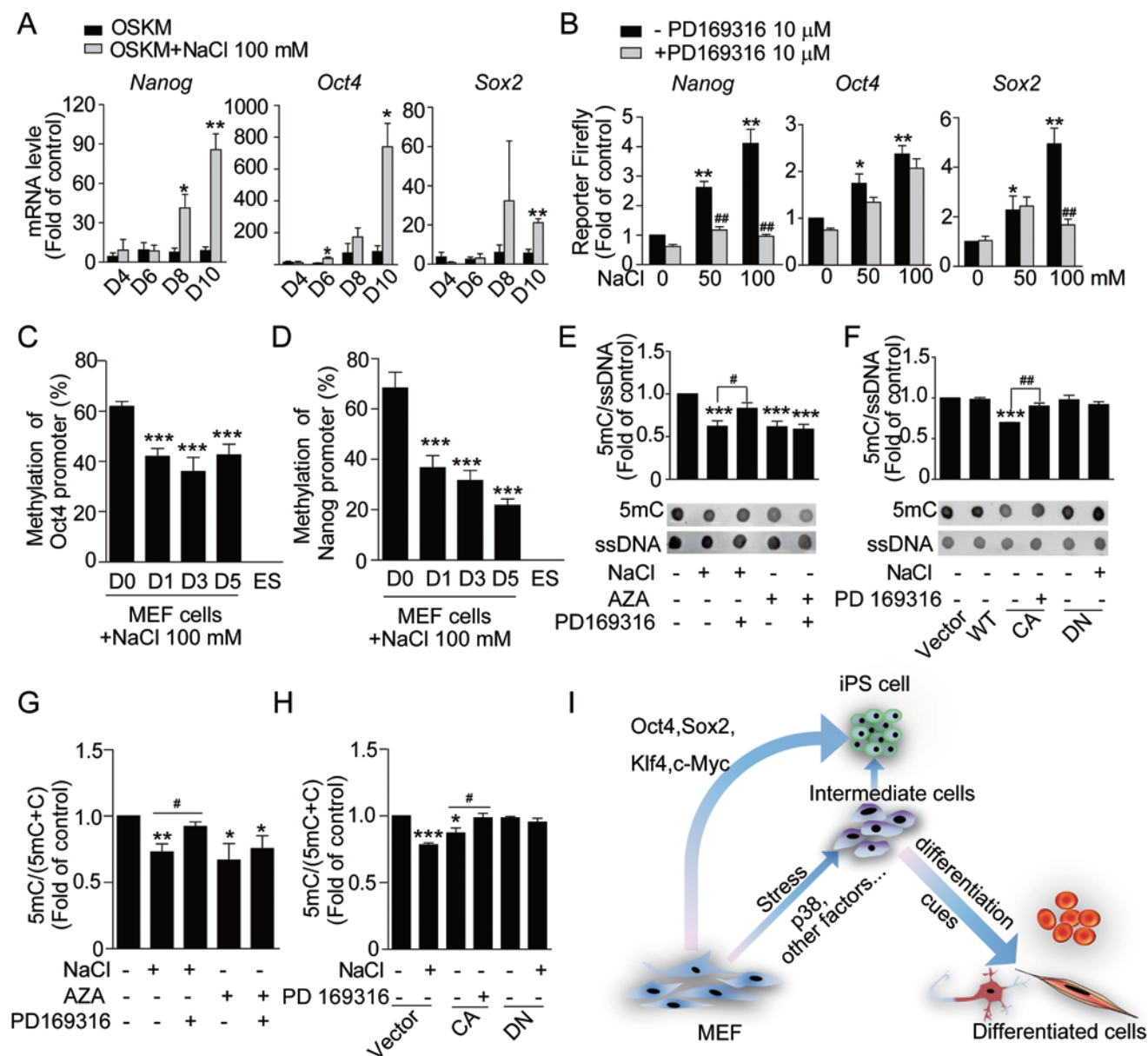
Figure 4 Stress mediated-p38 activation may promote reprogramming by reducing the global DNA methylation level. **(A)** qPCR analysis of gene expression during 4F-mediated reprogramming in the presence of NaCl or not. Expression levels were normalized to *GAPDH*, mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, versus no NaCl. **(B)** *Nanog*, *Oct4* and *Sox2* reporter activation by hyperosmosis in HEK293T cells in the presence of PD169316 (10 μM) or not. Mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, versus no NaCl, no PD169316 control; ## $P < 0.01$, versus no PD169316 group. **(C, D)** Bisulfite sequencing analysis of methylation levels at the proximal promoter regions of *Oct4* **(C)** and *Nanog* **(D)** in MEFs treated with NaCl for 1, 3 or 5 days. ES-14 was used as a control. Percent of the CpGs that were methylated are presented. Data are mean ± SEM of ten clones. *** $P < 0.001$, versus MEF without treatment (D0). **(E)** Dot blot analysis of 5-mC in DNA extracted from MEFs treated with NaCl (100 mM) or 5-Aza (10 μM) in the presence of PD169316 (10 μM) or not. The levels of 5-mC were normalized to total ssDNA. Mean ± SEM of six independent experiments. *** $P < 0.001$, versus control; # $P < 0.05$. **(F)** Dot blot analysis of 5-mC in DNA extracted from MEFs infected with WT-, CA- or DN-p38, and treated with NaCl (100 mM) or PD169316 (10 μM). *** $P < 0.001$, versus vector; ## $P < 0.01$. **(G)** HPLC quantification of genomic content of 5-mC relative to cytosine plus 5-mC in MEFs treated with NaCl (100 mM) or 5-Aza (10 μM) in the presence of PD169316 (10 μM) or not. Data are mean ± SEM of four independent experiments. * $P < 0.05$, ** $P < 0.01$, versus no treatment; # $P < 0.05$. **(H)** HPLC quantification of 5-mC in DNA extracted from MEFs infected with WT-, CA- or DN-p38, and treated with NaCl (100 mM) or PD169316 (10 μM). * $P < 0.05$, *** $P < 0.001$, versus vector control without NaCl treatment; # $P < 0.05$. **(I)** A schematic representation of stress-facilitated reprogramming.

NaCl (100 mM) treatment significantly reduced the DNA methylation level in MEFs. Similar level of demethylation was also observed in MEFs treated with the DNA methytransferase inhibitor 5-Aza (10 mM). P38 inhibitor PD169316 (10 µM) significantly reversed hyperosmosis-mediated DNA demethylation, though it did not block 5-Aza-induced DNA demethylation (Figure 4E). Interestingly, salt stress has also been reported to alter DNA methylation in certain plants [21]. In MEFs transfected with CA-p38, the 5mC level was also significantly lower compared to the control, and this effect was blocked by PD169316 (10 µM). The DN-p38 did not affect the 5mC level by itself, but it prevented NaCl-induced demethylation of DNA (Figure 4F). HPLC was also used to quantify the 5mC levels. As shown in Figure 4G, NaCl treatment significantly reduced the level of 5mC, and this effect was blocked by PD169316. 5-Aza also induced a similar level of demethylation in the genomic DNA of MEFs, but PD169316 could not block 5-Aza-induced DNA demethylation. Then we measured 5mC levels in MEF genomic DNA after forced expression of CA-p38 or DN-p38. CA-p38 significantly decreased 5mC level, and this decrease was blocked by PD169316. DN-p38 had no effect on the methylation level of genomic DNA, but it blocked NaCl-induced demethylation (Figure 4H). Taken together, these data indicate hyperosmotic stress might affect gene transcription by reducing the globel DNA methylation level, and this effect is mediated mainly via the p38 pathway.

Discussion

Animal development from fertilized, totipotent eggs to individuals is an epigenetically programmed process and was believed to be irreversible in mammals. The groundbreaking work by Yamanaka in 2006 demonstrated that ectopic expression of defined transcription factors could reprogram murine somatic cells to iPS cells [22]. Shortly after that, human iPS cells were generated with similar strategy [4, 23]. Many iPS cells were extensively characterized and are similar to ES cells in their gene expression patterns and chromatin signatures [24]; some mouse iPS cells can develop into individuals after tetraploid complementation, the most stringent pluripotency test available [25].

Apparently, reprogramming leads to the silencing of somatic genes and reactivation of pluripotency genes concomitant with the resetting of chromatin structure, though the identity of the major chromatin-modifying factors involved in this process remains largely unclear. Higher histone acetylation levels are generally associated with more open chromatin structure and elevated gene expression. Treatment with HDAC inhibitors such

as VPA and butyrate results in an enhancement of reprogramming. Methylation at histone H3K9 is a repressive chromatin modification that hinders reprogramming. The histone H3K9 methyltransferase G9a inhibitor BIX-01294 also enhances reprogramming [9]. DNA methylation is another crucial part of epigenetic regulation. DNA methylation at the fifth position of cytosine has the specific effect of reducing gene expression and plays critical roles in normal cellular differentiation and organismal development [26]. Blocking DNA methylation with DNA methyltransferase inhibitor 5-Aza has also been reported to facilitate iPS cell induction [9]. In this study we demonstrated that environmental stress such as hyperosmosis greatly facilitated iPS cell generation via activation of the p38 pathway. Further study indicates stress-mediated p38 activation might promote reprogramming by reducing the global DNA methylation level and enhancing the expression of pluripotency genes. Recently, hypoxia, another form of environmental stress, has also been reported to facilitate the generation of mouse and human iPS cells [27]. We found hypoxia (5% and 1% O_2) induced a slight but notable increase (~1.5-fold) in p38 phosphorylation (Supplementary information, Figure S4G and S4H). We speculate that p38 activation might play a role, but it is not likely to be the main mechanism of hypoxia-enhanced reprogramming. Other mechanisms, such as Hif-2α-mediated *Oct4* transcription [28], might play a more important role.

Dramatic change in gene expression is a major component of stress responses, and such change is achieved by the modulation of chromatin structure, transcription, mRNA stability and translation via intracellular signaling. Environmental stresses, such as osmostress, UV, heat stress, oxidative stress, heavy metals and chemicals, as well as physiological mediators, such as inflammatory cytokines, activate the highly conserved stress-activated protein kinases (SAPKs) pathways [29]. The p38 MAPKs are the mammalian orthologues of yeast SAPK Hog1, an osmo-sensing protein. Stresses with various sensing mechanisms converge through different MAP3Ks on p38, which in turn regulate the activity of downstream transcription factors and regulators of chromatin remodeling [3]. Hyperosmotic stress activates other signaling pathways in addition to p38. We also found hyperosmosis activated all three MAPK pathways, p38, JNK and ERK. It is interesting to note that only p38 activation contributed to the positive effect of hyperosmosis on reprogramming. In fact, the activation of the other two pathways was detrimental [15] to iPS cell induction even though the summation of the signaling under hyperosmosis led to a beneficial effect. The duration of p38 activation was also critical to the enhancement effect, as

prolonged treatment with hyperosmotic condition or constant activation of p38 eventually leads to less iPS cells. This is understandable since cells can quantify stress and activate a death program when tolerance limits are exceeded [30].

Enhancement of reprogramming via p38 activation was surprising since p38 activation had long been associated with proliferation inhibition and differentiation induction. P38 activation also leads to p53-mediated premature senescence [31], which has been reported to be the major roadblock of somatic cell reprogramming [32]. Nevertheless, we observed that hyperosmosis-induced p38 activation stimulated the expression of pluripotency genes with or without the treatment of Yamanaka factors. Several pluripotency genes, such as *Sox2* and *Myc*, have also been found to be upregulated in MEFs in a whole genome analysis of p38-mediated gene expression upon stress [33]. Thus we speculate that a more general mechanism, rather than the expression of specific genes, might underlie the effect of stress-activated p38 on reprogramming. We found stress-mediated p38 activation led to a reduction of global DNA methylation level similar to the treatment of 5-Aza, and rendered the cells to enter an epigenetically unstable intermediate state. With the presence of Yamanaka factors or various differentiation cues, these cells are then more readily to be reprogrammed into the pluripotent state or induced to differentiate (Figure 4I).

In summary, we found stress-mediated p38 activation led to a reduction of global DNA methylation level, and rendered the cells to enter an epigenetically unstable intermediate state, resulting in a higher reprogramming efficiency. Our data not only provide a simple and safe way to facilitate the generation of iPS cells, but also demonstrate how simple environmental stress like hyperosmosis helps to alter the fate of cells via intracellular signaling and epigenetic modulation.

Materials and Methods

Derivation of MEFs and cell culture

OG2 mice, which carry a transgenic *Oct4* promoter driven GFP reporter, were mated with C57 mice and MEF cells were isolated from E12.5 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 μg/ml streptomycin. Isolated MEF cells in early passages (up to passage 3) were used for further experiments.

Mouse iPS cell 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 (passage 1-3) 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 trypsinized 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). NaCl or other chemicals were added from day 3 with various durations.

GFP$^+$ colonies were scanned and counted using Acumen eX3 microplate scanner (TTP Labtech) or ImageXpress® Micro System (Moleculer Devices) from day 8 post infection. GFP$^+$ colonies were also trypsinized and then analyzed using a FACS Calibur (BD). GFP$^+$ cells were gated with a control signal from the PE channel and a minimum of 10 000 events were recorded.

Alkaline phosphatase and immunofluorescent staining

For alkaline phosphatase staining, iPS cells were fixed with 4% paraformaldehyde (PFA) in PBS for 45 seconds, rinsed once with PBS and detection was performed using a leukocyte alkaline phosphatase 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 PrimeScriptTM RT reagent kit (Takara, DRR037A) according to the manufacturer's protocol. Real-time PCR was performed using JumpStartTM TaqReady MixTM (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 45 cycles at an optimal annealing temperature. Primers sequences are supplied in Supplementary information, Table S1.

Bisulfite sequencing

Bisulfite sequencing was used to detect DNA methylation in the promoter regions of *Oct4* and *Nanog* as described [22]. In brief, genomic DNA was isolated and bisulfite conversion was performed in agarose beads as described [34]. Nested PCR was performed to amplify the promoter regions after bisulfite conversion and the PCR products were cloned into pMD19-T vectors (Takara). Ten randomly selected clones from each sample were sequenced

and analyzed. PCR primers are listed in Supplementary information, Table S2.

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-α-Tubulin (11H10) (1:10 000; CST 2125), rabbit anti-p38 (1:1 000; CST 9212), rabbit anti-p-p38(1:1 000; CST 9211), rabbit anti-JNK (1:1 000; CST 9258), rabbit anti-p-JNK(1:1 000; CST 4668), rabbit anti-ERK (1:1 000; CST 9102), rabbit anti-p-ERK(1:1 000; CST 4370), or rabbit anti-p-MK2 (1:1 000; CST 3316). 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 Western Lightning Ultra (Perkin Elmer) and ChemiDoc imaging system (Bio-Rad).

Teratoma formation, chimera production and germline transmission

About 1×10^6 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, hematoxylin and eosin 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.

Luciferase reporter assays

For *Nanog*, *Oct4* and *Sox2* reporter activitiy, plasmids encoding promoter regions of *Nanog* (50 ng), *Oct4* (100 ng) or *Sox2* (25 ng) together with a firefly luciferase reporter were cotransfected with 25 ng Renilla luciferase driven by TK-promoter (Promega) into HEK293T cells seeded on 96-well plate at a density of 40 000 per well using Fugene HD (Roche). Chemicals were added 18 h after transfection and firefly and renilla luciferase activities were measured 24 h after drug treatment with the Dual-Glo Luciferase Assay System (Promega) using an EnVision multilabel plate reader (Perkin Elmer).

Dots blot analysis of total DNA 5-mC

MEFs were incubated in medium supplemented with 100 mM NaCl for 72 h. Genomic DNA was isolated using the DNA Isolation Kit (Promega) according to the manufacturer's instructions and 200 ng DNA from each sample was dotted onto the nitrocellulose membrane (GE Amersham). Membrane was exposed to UV light for 5 min and then incubated in blocking buffer for 1 h at room temparature. Membrane was incubated overnight with the 5-mC antibody (Active motif 39649) in blocking buffer at 4 °C. After through washing, membrane was probed with HRP-conjugated secondary antibody and visualized using Western Lightning Ultra and ChemiDoc imaging system (Bio-Rad).

HPLC quantification of total DNA 5-mC

The genomic DNA was extracted by phenol/chloroform then treated with RNase A/T1 twice (50 µg RNase A and 1 000 U RNase T1 per 100 µg DNA), at least 4 h each time. The DNA was purified by phenol/chloroform, precipitated in 70% ethanol, and dissolved in water. Approximately, 40 µg DNA was hydrolyzed with 0.1 U of nuclease P1 (Sigma) at 50 °C for at least 1 h or at 37 °C overnight (the reaction volume is 80 µl, containing 20 mM NaOAc, pH 5.3 and 0.2 mM $ZnSO_4$). 8 µl of 10× CIAP buffer and 1 µl of calf intestinal alkaline phosphatase (TaKaRa) were then added to the reaction followed by incubation for at least 2 h or overnight at 37 °C. The reaction products were then analyzed on an Agilent 1200 HPLC instrument with an AQ-C18 column (5-µm particle size, 25 cm × 4.6 mm). The mobile phase was 10 mM KH_2PO_4, pH 3.7, running at 1 ml per min, and the detector was set at 280 nm. Standard compounds 2′-deoxycytidine and 5-methyl 2′-deoxycytidine were bought from Sigma and TCI.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)