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Induction of pluripotent stem cells from primary human fibroblasts with only *Oct4* and *Sox2*

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Ectopic expression of defined sets of genetic factors can reprogram somatic cells to induced pluripotent stem (iPS) cells that closely resemble embryonic stem (ES) cells. The low efficiency with which iPS cells are derived hinders studies on the molecular mechanism of reprogramming, and integration of viral transgenes, in particular the oncogenes *c-Myc* and *Klf4*, may handicap this method for human therapeutic applications. Here we report that valproic acid (VPA), a histone deacetylase inhibitor, enables reprogramming of primary human fibroblasts with only two factors, *Oct4* and *Sox2*, without the need for the oncogenes *c-Myc* or *Klf4*. The two factor–induced human iPS cells resemble human ES cells in pluripotency, global gene expression profiles and epigenetic states. These results support the possibility of reprogramming through purely chemical means, which would make therapeutic use of reprogrammed cells safer and more practical.

Patient-specific stem cells may be created by reprogramming somatic cells to a pluripotent state. A somatic cell can be reprogrammed by transferring its nucleus into an oocyte[1–4] or by fusion with an ES cell[5,6]. These studies demonstrate that pluripotency can be restored in a terminally differentiated cell and suggest that factors present in oocytes or ES cells can reprogram a somatic nucleus to a totipotent or pluripotent state. Recently, pioneering work by Yamanaka and colleagues identified key transcription factors that, when overexpressed, enable reprogramming of a somatic cell to a pluripotent state[7]. It is now possible to reprogram mouse and human somatic cells to a pluripotent state by ectopic expression of the factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* or a different set of four factors (*OCT4*, *SOX2*, *NANOG* and *LIN28*)[8–14]. Human iPS cells closely resemble ES cells in gene expression, pluripotency and epigenetic states, and hold great potential for regenerative medicine and *in vitro* disease modeling. More studies are needed to determine whether iPS cells and ES cells are equivalent in all aspects of function and potential.

Two issues appear to limit the utility of iPS cells: (i) the low efficiency of reprogramming primary human cells, which makes it difficult to generate patient-specific iPS cells from a small starting population of cells, and (ii) the integration of viral transgenes into the somatic genome, especially oncogenes such as *c-MYC* and *KLF4*. Indeed, reactivation of the *c-Myc* retrovirus contributes to tumor formation in chimeric mice derived from iPS cells[8,15]. Through optimization of the reprogramming method, it is now possible to reprogram both mouse and human somatic cells with the three-factor combination of *OCT4*, *SOX2* and *KLF4*, without the potent oncogene *c-MYC*[15,16]. However, three-factor reprogramming efficiency is low: fewer than one iPS colony was formed from 100,000 (<0.001%) infected human fibroblasts[15].

One approach proposed to circumvent these limitations is to use adult stem cells instead of differentiated cells as the starting population. Mouse neural stem cells that express endogenous Sox2 can be reprogrammed to a pluripotent state with the addition of only two transcription factors[17–19]. This approach, although potentially applicable for generating human iPS cells, is limited by the difficulty and potential risks of obtaining neural stem cells from patients' brains[20]. In addition, *Klf4*, a known oncogene, is still required in this approach. Another approach that we and others have explored is the use of small-molecule chemicals or growth factors in combination with the genetic factors to reprogram mouse somatic cells[18,21–23]. The goal of this approach is to replace the transfected genes with reagents that do not cause permanent changes to the somatic genome. For example, we have recently shown that histone deacetylase (HDAC) and DNA methyltransferase inhibitors greatly improve the efficiency of reprogramming mouse embryonic fibroblasts (MEFs) by genetic factors[21]. However, the usefulness of these small-molecule chemicals or growth factors in generating human iPS cells is unknown.

Here we show that valproic acid (VPA), a small-molecule HDAC inhibitor, greatly increases the efficiency of reprogramming primary human fibroblasts, a differentiated cell type, to a pluripotent state. In addition, VPA enables reprogramming of primary human fibroblasts with just two transcription factors, *Oct4* and *Sox2*. To our knowledge, the generation of human iPS cells with only two transcription factors has not been reported previously. Our results also show that small-molecule chemicals can replace at least some of the transcription factors used to reprogram differentiated human cells.

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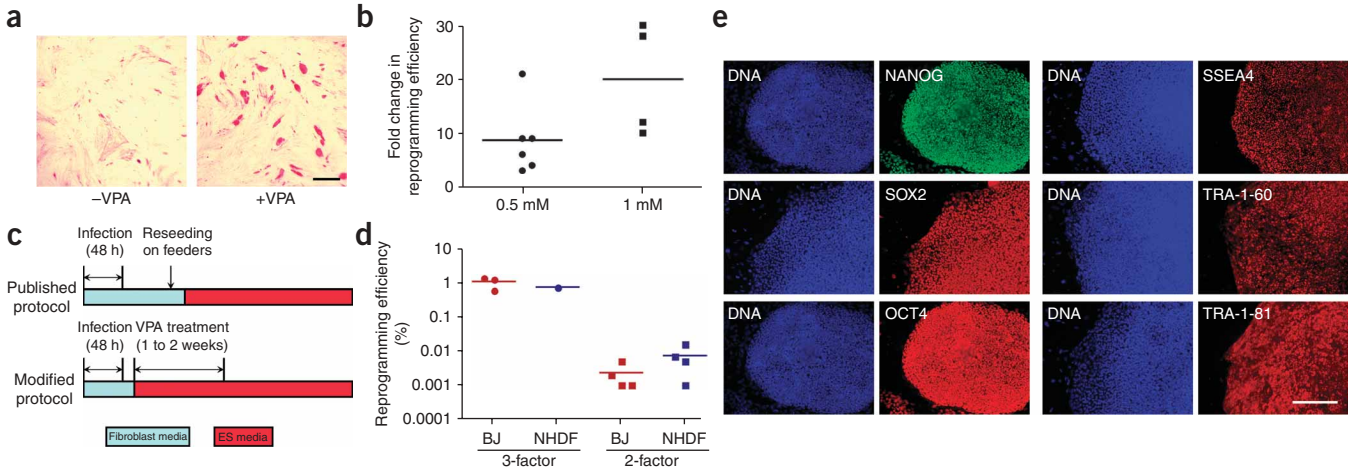

Figure 1 Efficient generation of iPS cells with three transcription factors (*Oct4*, *Sox2*, and *Klf4*). (**a**) ES cell–like colonies visualized by alkaline phosphatase staining were compared for three factor (*Oct4*, *Sox2* and *Klf4*)–infected BJ fibroblasts with or without VPA treatment at 25 d post-infection. (**b**) The fold change in reprogramming efficiency of VPA-treated BJ fibroblasts (0.5 mM and 1 mM) compared to untreated culture. Each dot represents one experiment and the bars represent the averages for each condition. (**c**) Schematic representations of the published three-factor reprogramming protocol[15] and the modified protocol. In the published protocol, infected human fibroblasts are cultured in fibroblast media first, then reseeded on feeders and switched to human ES-cell media. In our modified protocol, human fibroblasts were replated immediately after infection, treated with VPA in human ES-cell media and subsequently cultured in human ES-cell media. (**d**) The reprogramming efficiency by three factors (*Oct4*, *Sox2* and *Klf4*) and two factors (*Oct4* and *Sox2*) using the modified protocol with VPA treatment are plotted. Each dot represents one experiment; the bars represent the averages for each condition. iPS colonies were identified based on ES cell–like morphology in most experiments. In some two-factor reprogramming experiments (5 out of 8), the number of iPS colonies was determined by the number of iPS lines established from picking all ES cell–like colonies. (**e**) Immunofluorescence staining shows expression of pluripotency markers (OCT4, SOX2, NANOG, SSEA4, TRA-1-60 and TRA-1-81) in three factor–induced iPS cells. Scale bar, 1 mm for **a**, 250 μm for **e**.

RESULTS

Efficient induction of iPS cells with three factors using VPA and an optimized protocol

We have previously shown that VPA, an HDAC inhibitor, enables efficient induction of iPS cells from mouse fibroblasts infected by three factors, *Oct4*, *Sox2* and *Klf4* (~2% based on the induction of Oct4-GFP$^+$ cells)[21]. Here we examine the effect of VPA on primary human fibroblasts infected by the same three factors. We used two primary human fibroblast lines: BJ (neonatal human foreskin fibroblasts) and NHDF (normal neonatal human dermal fibroblasts). Human fibroblasts were first infected by Moloney murine leukemia retroviruses expressing the *Oct4*, *Sox2* and *Klf4* genes, treated with or without VPA for 1 to 2 weeks, and then cultured in human ES-cell media until ~1 month post-infection. In both BJ and NHDF, VPA increased the number of ES cell–like colonies by 10- to 20-fold when examined at ~1 month post-infection (**Fig. 1a**,**b** and **Supplementary Fig. 1** online). As has been reported for reprogramming mouse cells[24,25], human iPS colonies can be readily identified by ES cell–like morphology, and most ES cell–like colonies formed from infected human fibroblasts can be expanded and show expression of pluripotency markers[11–13,15]. To ensure that ES cell–like morphology is a reliable criterion for identifying iPS colonies in our three factor–infected human fibroblast culture, we examined 20 colonies (10 from cultures treated with VPA and 10 from untreated cultures) picked on the basis of their ES cell–like morphology. All colonies were expanded and immunofluorescence staining showed expression of pluripotency markers, including NANOG (**Supplementary Fig. 2** online). Thereafter, an ES cell–like morphology was used to identify putative iPS colonies, and the reprogramming efficiency was determined by the number of iPS colonies formed out of the total number of infected cells seeded, as reported previously[11–13,15].

We continued to optimize the reprogramming protocol in addition to the use of VPA. In the study on three-factor reprogramming of human fibroblasts[15], infected human cells were first cultured in serum-containing fibroblast media, reseeded on feeders, and then transferred to human ES-cell media ~1 week post-infection (**Fig. 1c**). Serum replacement, a component of typical human ES-cell media, has been shown to promote the reprogramming efficiency of mouse cells[25]. We therefore reasoned that switching to human ES-cell media containing serum replacement sooner might promote the efficiency of reprogramming human fibroblasts. We also reasoned that the cloning efficiency of dissociated cells undergoing reprogramming may be low, as shown for dissociated human ES cells (<1%)[26,27], so that replating infected human fibroblasts could decrease the efficiency of iPS colony formation. We therefore made the following modifications: (i) cells were seeded in fibroblast media immediately after the infection and switched to serum replacement–containing human ES-cell media the next day, and (ii) the infected cells were allowed to grow in human ES-cell media without splitting or replating onto feeder cells until iPS colonies could be picked, ~1 month post-infection. These changes substantially increased reprogramming efficiency (~30-fold) and, together with VPA treatment, led to a ~1% reprogramming efficiency based on the number of ES cell–like colonies formed from three factor–infected human fibroblasts (**Fig. 1d** and **Supplementary Table 1** online).

As mentioned above, iPS colonies from three factor–infected fibroblasts can be readily identified by their morphology and picked and expanded to establish iPS cell lines. The three factor–induced iPS cells closely resembled human ES cells in pluripotency marker expression (**Fig. 1e**), pluripotency and global gene expression profiles (described below). Thus, with VPA treatment and optimization of the reprogramming protocol, primary human fibroblasts can be

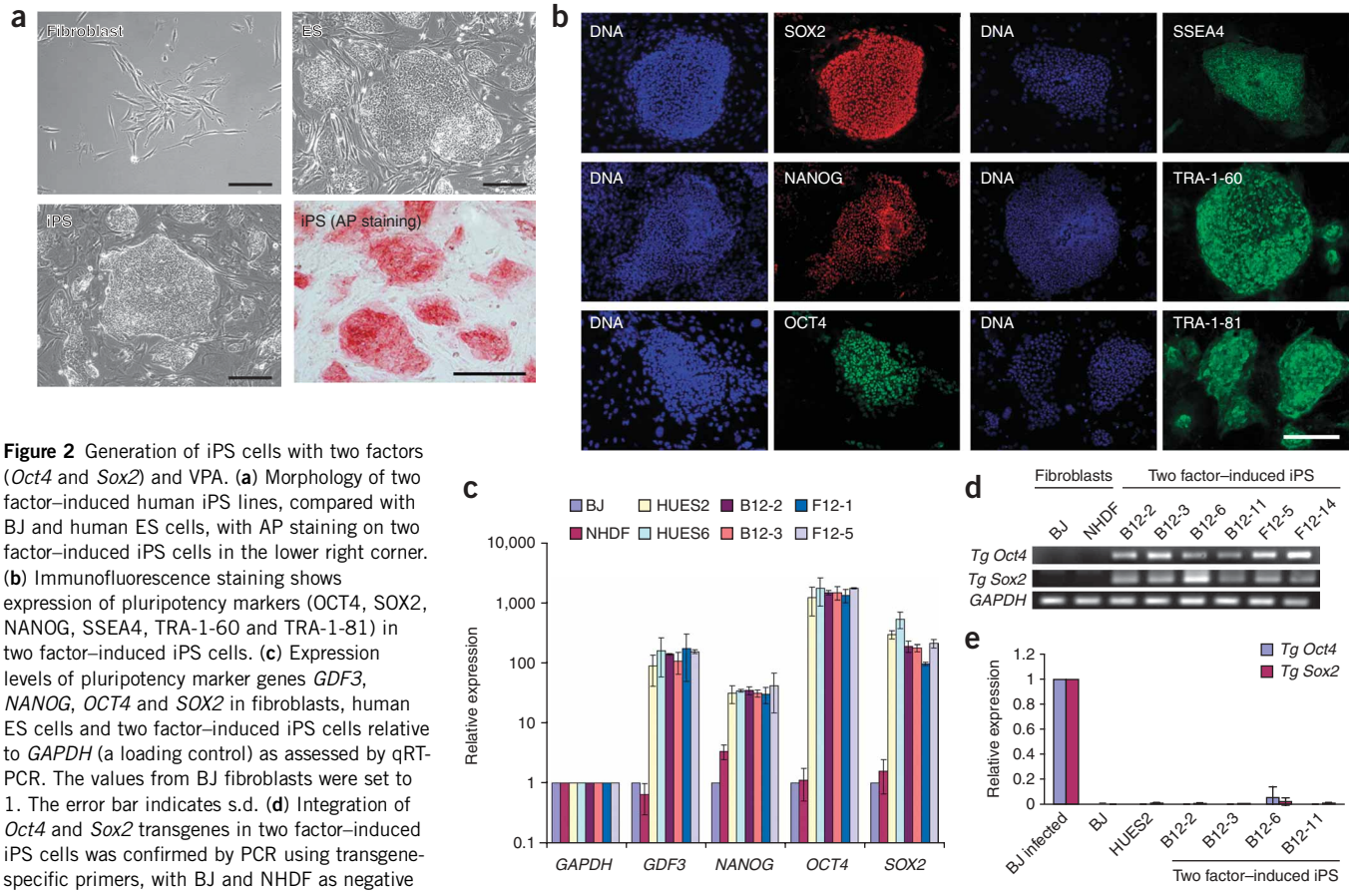

Figure 2 Generation of iPS cells with two factors (*Oct4* and *Sox2*) and VPA. (**a**) Morphology of two factor–induced human iPS lines, compared with BJ and human ES cells, with AP staining on two factor–induced iPS cells in the lower right corner. (**b**) Immunofluorescence staining shows expression of pluripotency markers (OCT4, SOX2, NANOG, SSEA4, TRA-1-60 and TRA-1-81) in two factor–induced iPS cells. (**c**) Expression levels of pluripotency marker genes *GDF3*, *NANOG*, *OCT4* and *SOX2* in fibroblasts, human ES cells and two factor–induced iPS cells relative to *GAPDH* (a loading control) as assessed by qRT-PCR. The values from BJ fibroblasts were set to 1. The error bar indicates s.d. (**d**) Integration of *Oct4* and *Sox2* transgenes in two factor–induced iPS cells was confirmed by PCR using transgene-specific primers, with BJ and NHDF as negative controls. *GAPDH* was used as a loading control. (**e**) Expression levels of transgenes *Oct4* and *Sox2* (relative to *GAPDH*) were assessed by qRT-PCR. The values from the infected BJ fibroblasts (isolated 7 d post-infection) were set to 1. Uninfected BJ and human ES cells (HUES2) were used as negative controls. The error bar indicates s.d. Scale bar, 250 μm.

reprogrammed efficiently by three transcription factors (*Oct4*, *Sox2* and *Klf4*). Compared to the one published report on reprogramming primary human cells by the same three-factor combination (efficiency <0.001%)[15], the approach described here improves reprogramming efficiency by ~1,000-fold (**Supplementary Table 1**). We note, however, that direct comparison of reprogramming efficiencies is difficult because of differences in cell types and the efficiency of viral infection.

Induction of human iPS cells with two factors

We next investigated whether iPS cells can be generated from primary human fibroblasts with fewer transcription factors. The optimized induction method with VPA treatment (**Fig. 1c**) was applied to human BJ and NHDF cells infected by different two-factor combinations. ES cell–like iPS colonies were identified ~1 month post-infection in fibroblasts infected with *Oct4* and *Sox2* but not with other two-factor combinations. iPS cell lines could be readily established by picking ES cell–like colonies formed from human fibroblasts infected by *Oct4* and *Sox2*. On average, between one and five iPS lines were successfully established out of every 100,000 BJ or NHDF cells infected by *Oct4* and *Sox2* (**Fig. 1d** and **Supplementary Table 1**). Thus, the two-factor reprogramming efficiency by VPA treatment is similar to the published induction rate for human fibroblasts infected by three factors (*OCT4*, *SOX2* and *KLF4*)[15], suggesting that VPA treatment effectively replaced the need for *KLF4*. We note, however, that on average, the efficiency of reprogramming by two factors with VPA treatment was significantly lower (~200-fold) than that by three factors with VPA treatment. Therefore, *KLF4*, although dispensable for reprogramming, plays an important facilitating role, as has been described for *c-MYC*[15,16].

The two factor–induced human iPS cells were readily cultured on irradiated MEF feeder cells in standard human ES-cell culture media without further VPA treatment. The iPS cells were karyotypically normal (**Supplementary Table 2** online), and DNA fingerprinting analysis confirmed their fibroblast origin (**Supplementary Table 3** online), excluding the possibility that they arose from contaminating human ES cells in the laboratory. The two-factor induced human iPS cells were morphologically similar to human ES cells and stained positive for alkaline phosphatase (**Fig. 2a**). Immunofluorescence staining confirmed expression of pluripotency markers in these iPS cells, including NANOG, OCT4, SOX2, SSEA4, TRA-1-60 and TRA-1-81 (**Fig. 2b**). Quantitative RT-PCR analysis showed that mRNA expression levels of pluripotency marker genes, including *GDF3*, *OCT4*, *NANOG* and *SOX2*, in two factor–induced iPS cells were similar to those of human ES cells and were markedly elevated compared with those of the parental fibroblast cells (**Fig. 2c**). In these assays, the *OCT4* and *SOX2* mRNAs were transcribed from endogenous *OCT4* and *SOX2* loci, which can be distinguished from the viral transgenes that express murine *Oct4* and *Sox2*. Genomic integration of the *Oct4* and *Sox2* transgenes was confirmed by PCR analysis, and multiple integration sites of the *Sox2* transgene were also detected by Southern blot analysis (**Fig. 2d** and **Supplementary Fig. 3** online). Variable degrees of transgene silencing have been reported

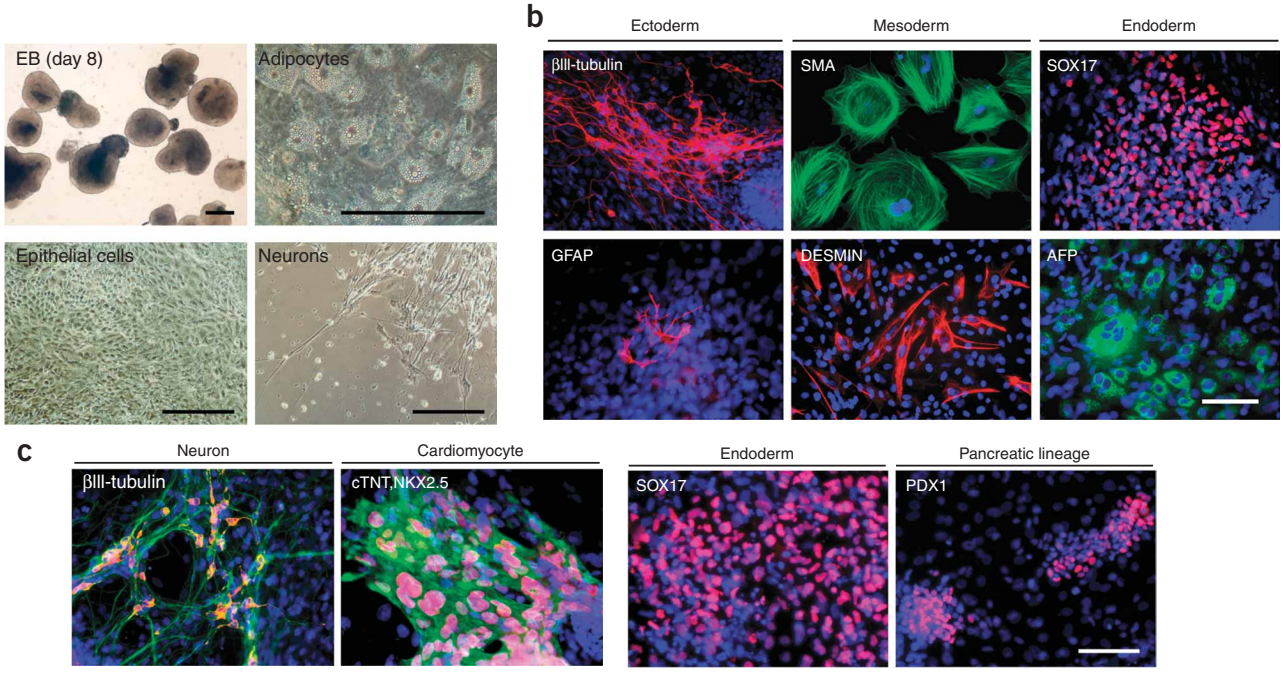

Figure 3 *In vitro* differentiation of two factor–induced iPS cells. (**a**) Two factor–induced iPS cells form embryoid bodies in suspension culture. Different cell types including adipocytes, epithelial cells and neurons can be identified by morphology after embryoid bodies were allowed to differentiate further in adherent culture. EB, embryoid body. (**b**) Immunofluorescence staining shows differentiation of two factor–induced iPS cells into cells expressing markers characteristic of the three germ layers. (**c**) Immunofluorescence staining shows differentiation of two factor–induced iPS cells to putative dopaminergic neurons (co-expression of βIII-tubulin in green and TH in red), cardiomyocytes (co-expression of cTNT (cardiac troponin) in green and NKX2.5 in red), definitive endoderm (SOX17) and pancreatic cells (PDX1). Scale bars, 250 μm for **a**, and 100 μm for **b** and **c**.

with human iPS cells generated in different laboratories[11–14]. We examined the expression of viral transgenes by quantitative RT-PCR and showed that the viral *Oct4* and *Sox2* transgenes were efficiently silenced in all iPS cell lines examined (**Fig. 2e**), indicating that the maintenance of two factor–induced iPS cells is independent of continued transgene expression.

In vitro differentiation of two factor–induced iPS cells

The ability of ES cells to differentiate into all cell types is the basis for their potential in regenerative medicine. We examined the differentiation capacity of the iPS cells *in vitro* (experimental scheme shown in **Supplementary Fig. 4** online). Like human ES cells, the two factor–induced iPS cells form embryoid bodies in suspension culture (**Fig. 3a**). Spontaneous differentiation of iPS cells was evident when these embryoid bodies were allowed to grow in adherent culture: epithelial cells, adipocytes and neurons (**Fig. 3a**), as well as beating cardiomyocytes (data not shown), were identified by morphology. Immunofluorescence staining and RT-PCR analysis confirmed differentiation of the two factor–induced iPS cells into derivatives of the three embryonic germ layers (**Fig. 3b**, **Supplementary Fig. 5a** online). We next examined whether directed differentiation of two factor–induced iPS cells could be induced through protocols established for

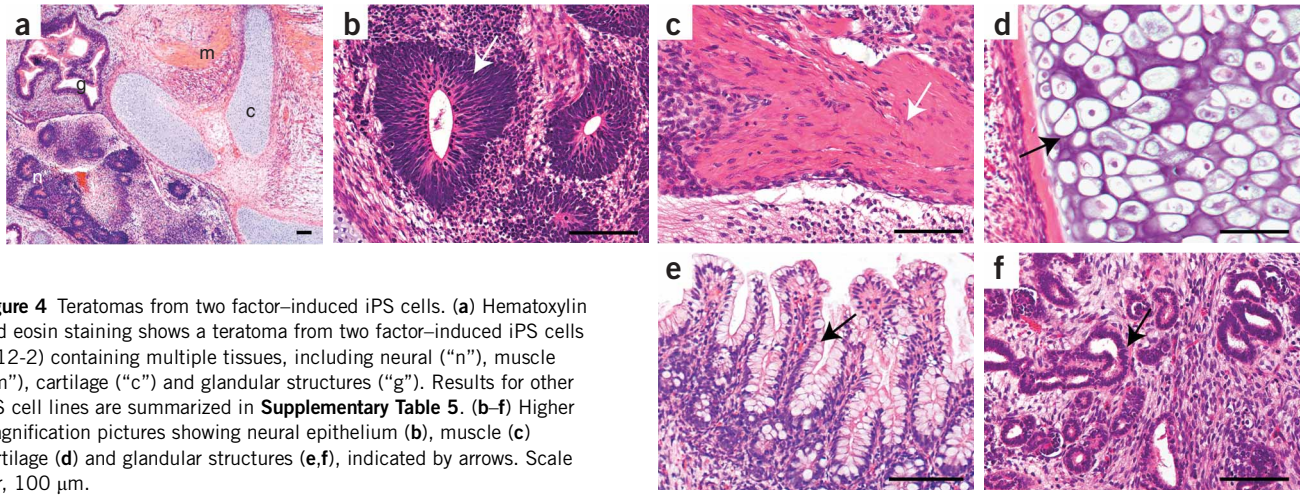

Figure 4 Teratomas from two factor–induced iPS cells. (**a**) Hematoxylin and eosin staining shows a teratoma from two factor–induced iPS cells (B12-2) containing multiple tissues, including neural ("n"), muscle ("m"), cartilage ("c") and glandular structures ("g"). Results for other iPS cell lines are summarized in **Supplementary Table 5**. (**b**–**f**) Higher magnification pictures showing neural epithelium (**b**), muscle (**c**) cartilage (**d**) and glandular structures (**e**,**f**), indicated by arrows. Scale bar, 100 μm.

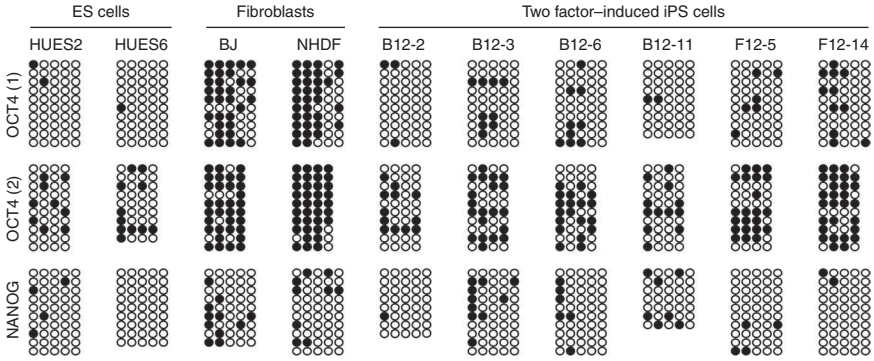

Figure 5 Methylation analysis of *OCT4* and *NANOG* promoter regions in two factor–induced iPS cells. Genomic DNA from human ES cells, fibroblasts and two factor–induced iPS cells were processed for bisulfite modification. The promoter regions of *OCT4* and *NANOG* were amplified by PCR using primer sets previously described[13,32,33], and methylation states were analyzed. Each horizontal row of circles represents an individual sequencing result from one amplicon. Open and filled circles indicate unmethylated and methylated CpG dinucleotides, respectively.

human ES cells. Two factor–induced iPS cells were successfully differentiated into neurons (ectoderm derivative) co-expressing βIII-tubulin and TH (tyrosine hydroxylase), the latter being a marker for dopaminergic neurons, beating cardiomyocytes (mesoderm derivative) and definitive endoderm as well as endoderm-derivative pancreatic cells following established protocols for these cell types[28–30]. Expression of markers characteristic of these differentiated cell types was confirmed by immunofluorescence staining and RT-PCR analysis (**Fig. 3c** and **Supplementary Fig. 5b–d**). These studies showed that the two factor–induced iPS cells were pluripotent and appeared to respond to the same signals that direct human ES cell differentiation.

Teratoma formation from two factor–induced iPS cells

Human ES cells form teratomas when injected into immunocompromised mice[27], which has become a standard assay of pluripotency. Similarly, the two factor–induced human iPS cells formed teratomas after subcutaneous injection into immunocompromised nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice. Histological examination of the teratomas revealed representative tissues originating from the three embryonic germ layers, including neural epithelium, muscle, cartilage and various glandular structures (**Fig. 4**). Thus, like human ES cells, the two factor–induced iPS cells have the capacity to differentiate both *in vitro* and *in vivo*. In addition, similar *in vitro* and *in vivo* differentiation experiments on three factor–induced iPS cell lines (**Supplementary Figs. 5** and **6** online) showed no significant differences in differentiation capacity between two factor– and three factor–induced iPS cells.

DNA methylation and global gene expression profiles of two factor–induced iPS cells

To further compare the two factor–induced iPS cells with human ES cells[31], we examined epigenetic modifications and global gene expression profiles. Similar to reprogramming through nuclear transfer or cell fusion, reprogramming through gene transduction is accompanied by epigenetic remodeling of the genome, including demethylation in promoters of pluripotent genes[11–13]. We therefore analyzed methylation states of CpG dinucleotides in the *OCT4* and *NANOG* promoter regions[13,32,33]. Bisulfite genomic sequencing analyses showed that *OCT4* and *NANOG* promoter regions were demethylated in two factor–induced iPS cells relative to the parental fibroblast lines and were similar to those of human ES cells (**Fig. 5**). These data are consistent with epigenetic remodeling during reprogramming by gene transduction.

Consistent with the quantitative RT-PCR results, microarray analysis showed that mRNA expression levels of pluripotency marker genes, including *GDF3*, *OCT4*, *NANOG* and *SOX2*, in both two factor– and three factor–induced iPS cells were similar to those in human ES cells and markedly elevated compared with those in fibroblasts (**Fig. 6a**). Hierarchical clustering analysis showed that iPS cells clustered together with human ES cells and were distinct from the parental fibroblast lines (**Fig. 6b**). Similarly, scatter plot analysis showed a tight correlation in gene expression between iPS cell and human ES cells (**Fig. 6c**). The linear coefficient of determination (r^2, the square of the correlation coefficient) values between iPS cells (or human ES cells) and fibroblasts were ~0.76. In contrast, the r^2 values

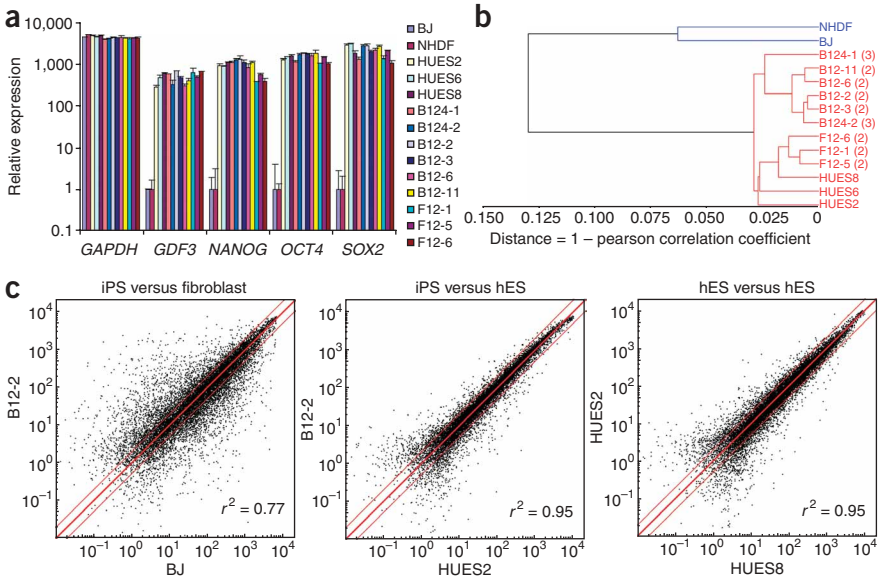

Figure 6 The gene expression profile of two factor–induced iPS cells closely resembles that of human ES cells. (**a**) The relative expression of *GAPDH*, *GDF3*, *NANOG*, *OCT4* and *SOX2* in fibroblasts, human ES cells and iPS cells by microarray analysis. (**b**) Hierarchical cluster analysis of the microarray data from human ES cells, fibroblasts and iPS cells. The numbers in parenthesis indicate the number of transcription factors used for the induction of different iPS lines. (**c**) Scatter plots comparing global gene expression profiles between two factor–induced iPS cells and fibroblasts, two factor–induced iPS cells and human ES cells, and two different human ES cell lines. The red lines indicate the linear equivalent and twofold differences on either side in gene expression levels. hES, human ES cells.

were ~0.95 between various two factor–induced iPS and human ES cell lines, similar to the r^2 values between different human ES cell lines (**Fig. 6c** and **Supplementary Table 4** online). This indicates that the difference between human iPS and ES cells is no greater than the difference between different human ES cell lines in global gene expression profiles. We conclude that two factor–induced iPS cells closely resemble human ES cells in global gene expression profiles as well as pluripotency and epigenetic state.

DISCUSSION

In summary, these experiments support two conclusions. First, VPA, an HDAC inhibitor, increases reprogramming efficiency of human fibroblasts, enabling a ~1% reprogramming efficiency of primary human fibroblasts infected with the transcription factors *Oct4*, *Sox2* and *Klf4*. This reasonably high efficiency of reprogramming may allow for the generation of patient-specific iPS cells from a small starting population of cells and may facilitate mechanistic studies, such as detection of molecular changes during the reprogramming process. The effect of VPA and other HDAC inhibitors on reprogramming[21] suggests that chromatin remodeling is a rate-limiting step in the process.

The second conclusion begins to address concerns surrounding the integration of viral transgenes into the somatic genome[34–36], in particular, the oncogenes *c-MYC* and *KLF4*. It has been shown recently that mouse neural stem cells, which express endogenous Sox2, can be reprogrammed with two transcription factors[17–19]. Here we show that two transcription factors plus VPA are sufficient to generate iPS cells from primary human neonatal fibroblasts, a differentiated cell type that is not already expressing Sox2 or other known reprogramming factors. Our findings are probably applicable to readily obtainable cells from adult patients, such as skin fibroblasts. In addition, elimination of the oncogenes *c-MYC* and *KLF4* is likely to be preferred for therapeutic use of reprogrammed cells. The use of VPA for the generation of iPS cells does not appear to affect the ability of the cells to self-renew or differentiate both *in vitro* and *in vivo*. In addition, VPA is a US Food and Drug Administration–approved treatment for epilepsy[37] and may therefore be readily applied in a clinical setting.

The current study is a step forward in defining core factors for the induction of pluripotency from differentiated human fibroblasts. Studies using mouse neural stem cells suggest that *Klf4* is essential for reprogramming mouse cells to a pluripotent state[17–19]. Our findings, together with previous studies[11–14], show that although *Klf4* plays a facilitating role, it is dispensable for reprogramming human cells to a pluripotent state. *OCT4* and *SOX2* are both part of the core regulatory circuitry of pluripotency[38], and they are, so far, the only common factors used by different researchers to induce pluripotent stem cells from primary human cells[11–14]. We show here that in the presence of VPA, *OCT4* and *SOX2* are sufficient for reprogramming human fibroblasts, providing further support for their central roles in the induction of pluripotency. Our results raise the question of whether, or how soon, it will be possible to find small molecules to replace *OCT4* and *SOX2* and thereby achieve reprogramming through purely chemical means, making therapeutic use of reprogrammed cells safer and more practical.

METHODS

Cell culture. Human BJ (ATCC) and NHDF (Lonza Biosciences) cells were maintained in fibroblast medium: DMEM/M199 (4:1) supplemented with 15% FBS, L-glutamine and penicillin/streptomycin. Human ES and iPS cells were maintained on irradiated MEF feeder cells in human ES cell media. Knockout DMEM supplemented with 10% knockout serum replacement, 10% human plasma fraction, 10 ng/ml bFGF, nonessential amino acids, β-mercaptoethanol, L-glutamine and penicillin/streptomycin as previously described[31].

Retrovirus production. Moloney-based retroviral vectors (pMXs) containing the murine and human complementary DNAs (cDNAs) of *Oct4*, *Sox2* and *Klf4* (refs. 7,11) were obtained from Addgene. These plasmids were co-transfected into 293T cells with packaging vectors (pUMVC and pCMV-VSVG), and viral supernatants were collected 48 and 72 h post-transfection to infect human fibroblasts. Two to four rounds of infection were performed during a 48-h period. The typical infection efficiency is 70–90%, judging by expression of a control GFP vector or immunofluorescence staining for expression of Oct4 or Sox2. The day that viral supernatants were removed was defined as 0 day post-infection. Although most of our experiments were performed using the murine cDNAs, similar effects of VPA were observed on human fibroblasts infected by human cDNAs of *OCT4*, *SOX2* and *KLF4* (**Supplementary Fig. 1**).

Induction of pluripotent stem cells. For the generation of two factor–induced iPS cells, human fibroblasts were infected by *Oct4* and *Sox2*, and replated in fibroblast medium typically at 1–2 × 10^5 cells per well in gelatin-coated 6-well plates at 0 day post-infection. Cells were cultured in human ES-cell media starting from 1 d post-infection. Treatment with VPA (0.5–1 mM) begins typically at 1 d post-infection and lasts for up to 2 weeks. Treatment with 0.5 mM VPA for 10 d appears optimal for iPS colony formation from two factor–infected human fibroblasts. Generation of iPS cells with 3 factors was done either with the above protocol or the published protocol[15] with or without VPA treatment.

To establish iPS cell lines, iPS colonies were picked about 1 month post-infection based on ES cell–like colony morphology. The picked colonies were subsequently expanded and maintained on irradiated MEF feeder layers in human ES-cell media without VPA. Y-27632, a ROCK inhibitor that enhances survival of dissociated single human ES and iPS cells[13,39], was used at 5–10 μM to increase the seeding efficiency of iPS cells for the initial colony expansion after picking and for the first 2 d after each passaging. Three factor (*Oct4*, *Sox2* and *Klf4*)–induced iPS cells from BJ and NHDF fibroblasts were named as "B124-" and "F124-", respectively, followed by a number to distinguish between different clones. Similarly, two factor (*Oct4* and *Sox2*)–induced iPS lines from BJ and NHDF fibroblasts were named as "B12-" and "F12-", respectively, followed by a number. Characterization of these iPS cell lines, including marker expression and pluripotency analysis was summarized in **Supplementary Table 5** online.

VPA and Y-27632 were purchased from EMD Biosciences, and stock solutions were made in media. Karyotyping of the iPS cell lines was performed by the Clinical & Research Cytogenetics Laboratories at the Oregon Health & Sciences University. DNA fingerprinting analysis was performed by Cell Line Genetics.

Determining reprogramming efficiency. Reprogramming efficiency was calculated as the number of iPS colonies formed per number of infected cells seeded. iPS colonies were identified based on ES cell–like morphology, and alkaline phosphatase staining was used to facilitate the identification of iPS colonies. In some experiments, the number of iPS colonies was scored by the number of iPS cell lines established by picking all ES cell–like colonies. Similar results were obtained by these two scoring methods.

***In vitro* differentiation of human iPS cells.** For spontaneous differentiation through embryoid body formation, human iPS cells were dissociated by collagenase IV treatment and transferred to low attachment 6-well plates in knockout DMEM supplemented with 20% knockout serum replacement, nonessential amino acid, β-mercaptoethanol, L-glutamine and penicillin/streptomycin. After 8 d in suspension culture, embryoid bodies were transferred to gelatin-coated plates and cultured in the same medium for another 8 d.

Established protocols for directed differentiation of human ES cells were used for the differentiation of human iPS cells into putative dopaminergic neurons and cardiomyocytes[28,29]. For induction of definitive endoderm cells, an established protocol for human ES cells using activin A treatment[30] was employed. Briefly, undifferentiated human iPS cells at ~80% confluence were induced to differentiate into definitive endoderm cells with 100 ng/ml recombinant activin A (R&D) in RPMI1640 medium supplemented with 2% FCS, L-glutamine and penicillin/streptomycin for 4 d. For the induction of pancreatic progenitor cells, activin A-treated cells were cultured for 8 additional days in DMEM/F12 supplemented with N2 and B27 supplements, nonessential

amino acids, β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, L-glutamine and penicillin/streptomycin.

Teratoma formation by human iPS cells. Human iPS cells grown on MEF feeder layers were collected by collagenase IV treatment and injected subcutaneously into NOD-SCID mice. Palpable tumors were observed typically 1–2 months after injection. Tumor samples were collected typically in 2–3 months and processed for paraffin embedding and hematoxylin and eosin staining following standard procedures.

Alkaline phosphatase and immunofluorescence staining. AP staining was performed with the Vector Red substrate kit from Vector Laboratories. Immunofluorescence staining was performed using the following primary antibodies: AFP (Sigma), cTNT (NeoMarkers), DESMIN (Lab Vision), GFAP (DAKO), NANOG (R&D Systems), NKX2.5 (Santa Cruz Biotechnology), OCT4 (Santa Cruz Biotechnology), PDX1 (R&D systems), SMA (Sigma), SSEA4 (Chemicon), SOX2 (Santa Cruz Biotechnology), SOX17 (R&D systems), TH (Chemicon), TRA-1-60 (Chemicon), TRA-1-81 (Chemicon), βIII-tubulin (Covance Research Products).

Whole-genome expression analysis. For transcriptional analysis, total RNA was isolated from cells cultured in 6-well dishes usingRNeasy Mini Kit and QIAshredder from Qiagen. Biotinylated antisense RNA were amplified using Illumina Total Prep RNA amplification Kit from Ambion, hybridized to Illumina Whole-Genome Expression BeadChips (HumanRef-8) and analyzed by Illumina Beadstation 500. All samples were prepared in two to three biological repeats. Data were analyzed using the Beadstudio software provided by Illumina. Negative expression values (due to Beadstudio normalization) were reset to 1 for plotting relative expression levels in the log scale.

Bisulfite genomic sequencing. Genomic DNA (1 μg) from various cell lines were processed for bisulfite modification using CpGenome Universal DNA Modification Kit (Chemicon). The promoter regions of *OCT4* and *NANOG* were amplified by PCR using primer sets previously described[13,32,33]. Primer sequences are provided in **Supplementary Table 6** online. The PCR products were cloned into pCRII-TOPO vector using TOPO TA cloning kit (Invitrogen) and sequenced.

RT-PCR and PCR. Total RNA was isolated using RNeasy kit (Qiagen) followed by cDNA synthesis using Superscript III Reverse Transcriptase and Oligo (dT)12-18 primers (Invitrogen). PCR was performed with JumpStart Taq DNA polymerase (Sigma). Quantitative PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen) and analyzed with MJ-Opticon. Primer sequences were supplied in **Supplementary Table 6**.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

D.H. and D.A.M. conceived the experiments and wrote the manuscript. D.H., K.O., R.M., W.G., A.E., S.C. and W.M. performed experiments.

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