449

Induced Pluripotent Stem Cell Generation using Nonviral Vector

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ABSTRACT

Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by etopic expression of transcription factors. iPS cells are indistinguishable from ES cells in terms of morphology and stem cell marker expression. Moreover, mouse iPS cells give rise to chimeric mice that are competent for germline transmission. However, mice derived from iPS cells often develop tumors. Furthermore, the low efficiency of iPS cell generation is a big disadvantage for mechanistic studies. Nonviral plasmid-based vectors are free of many of the drawbacks that constrain viral vectors. The histone deacetylase inhibitor valproic acid (VPA) has been shown to improve the efficiency of mouse and human iPS cell generation, and vitamin C (Vc) accelerates gene expression changes and establishment of the fully reprogrammed state. The MEK inhibitor PD0325901 (Stemgent) has been shown to increase the efficiency of the reprogramming of human primary fibroblasts into iPS cells. In this report, we described the generation of mouse iPS cells devoid of exogenous DNA by the simple transient transfection of a nonviral vector carrying 2A-peptide-linked reprogramming factors. We used VPA, Vc, and the MEK inhibitor PD0325901 to increase the reprogramming efficiency. The reprogrammed somatic cells expressed pluripotency markers and formed EBs.

(Key words : Embryonic stem cell, Induced pluripotent stem cell, Nonviral vector)

INTRODUCTION

Stem cells are undifferentiated cells that can differentiate into diverse specialized cell types and self-renew to produce more stem cells (Burns and Zon, 2002; Morrison *et al.*, 1997). They fall into three categories in terms of potency (their ability to differentiate into other cell types) (Czyz *et al.*, 2003; gilbert, 1994). Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of the blastocyst whose isolation was first reported in the early 1980s (Evans and Kaufman, 1981; Martin, 1981). ES cells have the capacity to give rise to all somatic cell types (Donovan and Gearhart, 2001) and thus are expected to contribute greatly to regenerative medicine. However, the two most important issues associated with ES cells are immune rejection and medical ethics (Lamba *et al.*, 2009; Liu *et al.*; Mandai *et al.*).

Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by forced expression of defined factors (Takahashi and Yamanaka, 2006). iPS cells are indistinguishable from ES cells in terms of morphology and stem cell marker expression. Moreover, mouse iPS cells give rise to chimeric mice that are competent for germline transmission (Maherali *et al.*, 2007; Okita, *et al.*, 2007; Wernig, *et al.*, 2007). However, mice derived from iPS cells often develop tumors because the retroviral integration of transcription factors can activate or inactivate host genes (Nienhuis *et al.*, 2006; Okita *et al.*, 2008; Takahashi and Yamanaka, 2006). Furthermore, the low efficiency of iPS cell generation is a big disadvantage for mechanistic studies (Silva *et al.*, 2008; Takahashi and Yamanaka, 2006).

Nonviral plasmid-based vectors are free of many of the drawbacks that constrain viral vectors (Hengge *et al.*, 1995; Hengge *et al.*, 1996). In addition, efficient multiprotein expression has been reported in a variety of cell types using the 2A peptide sequence of foot and

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mouth disease virus (F2A) or 2A- like sequences from other viruses (Hasegawa *et al.*, 2007; Kaji *et al.*, 2009; Szymczak, *et al.*, 2004). Recently, this multiprotein expression strategy was also applied to reprogramming through transient transfection (Okita *et al.*, 2008). The histone deacetylase inhibitor valproic acid (VPA) has been shown to improve the efficiency of mouse and human iPS cell generation (Huangfu *et al.*, 2008a; Huangfu *et al.*, 2008b), and vitamin C (Vc) accelerates gene expression changes and establishment of the fully reprogrammed state (Esteban *et al.*, 2010). The MEK inhibitor PD0325901 (Stemgent) has been shown to increase the efficiency of the reprogramming of human primary fibroblasts into iPS cells (Lin *et al.*, 2009).

Here we report that VPA, Vc, and the MEK inhibitor PD0325901 enable reprogramming of primary mouse fibroblasts with a nonviral plasmid vector. The results of alkaline phosphatase (AP) staining and immunocytochemical analysis indicate that nonviral-induced mouse iPS cells resemble mouse ES cells in terms of pluripotency.

EXPERIMENTAL PROCEDURES

Mouse Embryonic Fibroblast Isolation and iPS Cell Culture

Mouse embryonic fibroblasts (MEFs) were isolated from day-13.5 embryos (E13.5) of C57BL/6 mice. MEFs were cultured in fibroblast growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin] for no more than six passages before plasmid transduction. iPS cells were cultured in mouse ES cell medium (Knockout DMEM supplemented with 20% Knockout Serum Replacer, l-glutamine, nonessential amino acids, 2-mercaptoethanol, 1% penicillin/streptomycin, and 1,000 U/ml leukemia inhibitory factors) with mitomycin C-treated MEF cells as feeder cells or on 0.1% gelatin-coated dishes. Vc (25 μg/ml) was added from day 6 until the end of each experiment unless otherwise indicated, and included in the medium used in the continuous culture of picked mouse iPS cell colonies. VPA (1 mM) was added from days 6 to 14 of mouse iPS cell generation. The MEK inhibitor PD0325901 was added to the medium on day 15 at a final concentration of 1 μM (Si-Tayeb *et al.*, 2010). The medium was changed daily.

Plasmid Preparation and Infection

Two types of vector were used: nonviral plasmid pCX expressing murine Oct4, Sox2, and Klf4 (Addgene) and nonviral plasmid pCX expressing murine c-Myc (Addgene) (Okita, *et al.*, 2008). MEFs were seeded to a 100-mm dish (1.4 ×10^6 cells per well) (day 0). Plasmids (3 μg) were introduced using 9 μl of FuGENE HD transfection reagent (Promega). From day 6, the transfected MEFs were cultured in mouse ES cell medium.

AP Staining

AP staining was performed using an alkaline phosphatase staining kit (Stemgent) according to the manufacturer's protocol.

Immunocytochemistry

Cells were fixed through incubation with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS; Gibco) for 20 min at room temperature (RT). Primary antibodies against Oct4 (diluted 1:100; Stemgent) and Nanog (1:100; Stemgent) were used in immunocytochemical analyses. These primary antibodies were detected with Alexa Fluor 594-conjugated anti-rabbit IgG (H+L) (Invitrogen).

Samples were incubated with blocking solution [10% normal goat serum (Vector), 0.1% Triton X-100 (Sigma) in DPBS (Gibco)] for 1 h at RT. Samples were then incubated overnight at 4℃ with primary antibodies in blocking solution. After three washes with DPBS, samples were incubated with secondary antibody for 1 h at RT in the dark. Each well was then washed three times with DPBS with gentle agitation (10 min per wash). Residual DPBS was aspirated from the wells and 1~2 drops of mounting medium containing DAPI (Vector) were added. Images were captured using an IX71 inverted research microscope (Olympus) using the same laser intensity and detection sensitivity.

Quantitative Real-Time Polymerase Chain Reaction (Real-Time PCR) Analysis

cDNAs were synthesized from ES cells and iPS cells. Real-time PCR was performed using a StepOnePlus™ PCR system (Applied Biosystems), which exploits the ability of SYBR green to fluoresce after hybridization with double-stranded DNA. The following primers were used: Oct4 forward, 5′-GCC CTC CCT ACA GCA GAT CA-3′; Oct4 reverse, 5′-GAA CCA TAC TCG AAC CAC ATC CT-3′; Sox2 forward, 5′-CCG ATG CAC CGC TAC GA-3′; Sox2 reverse, 5′-GGT GCC CTG CTG CGA GTA-3′; Nanog forward, 5′-TGG AAG CCA CTA GGG AAA GC-3′; and Nanog reverse 5′-TGG AGT CAC AGA GTA GTT CAG GAA TAA-3′. Real-time PCR was started with initial denaturation at 95℃ for 30 s, followed by 50 cycles of denaturation at 95℃ for 5 s, annealing at 60℃ for 30 s, and elongation at 72℃ for 10 s. The *GAPDH* gene was used as an internal control. Levels of cysteine cathepsins and GAPDH gene expression in all cDNA samples were determined from the level of SYBR green fluorescence using StepOne™ Software Version 2.1 (Applied Biosystems).

Embryoid Body Formation Analysis

ES and iPS cells were harvested and cultured without leukemia inhibitory factor (LIF) to initiate embryoid body (EB) formation. Then 2×10^6 cells were seeded as a suspension in10 ml of DMEM to a 100-mm low attachment dish (bacterial-grade). Numbers and sizes of EBs in each sample were determined by removing 200 µl of culture for light microscopic imaging using a DP 25 inverted research microscope (Olympus). EBs larger than 100 µm in diameter were counted.

Statistical Analyses

The results are expressed as the mean±SEM of at least three independent experiments. Groups were compared by Student's *t*-test. In all analyses, *p*<0.05 was taken to indicate statistical significance.

RESULTS

Induced Pluripotent Stem Cell Generation using a Nonviral Vector
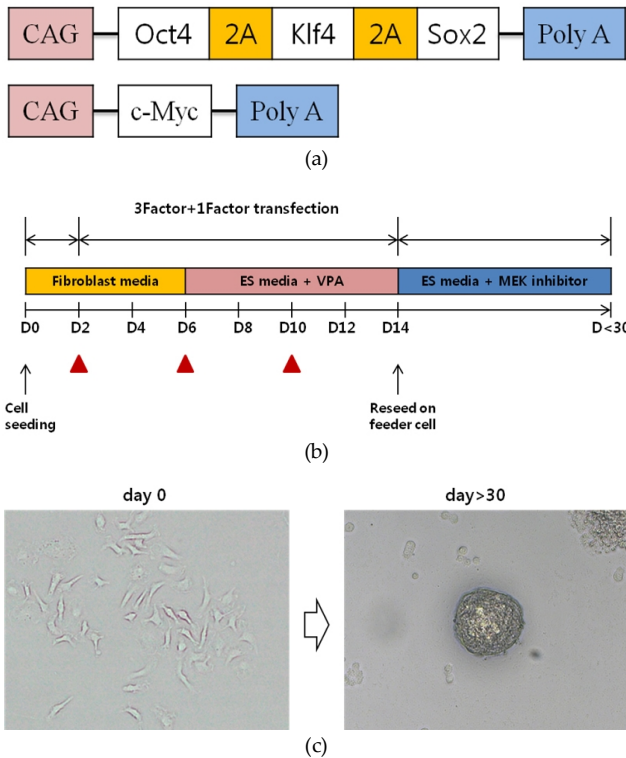

(a)

(b)

(c)

Fig. 1. Generation of iPS cells using nonviral vectors. (a) Expression plasmids for iPS cell generation. cDNAs encoding Oct4, Klf4, and Sox2 were connected in this order with the 2A peptide and inserted into the pCX plasmid [15]. In addition, a c-Myc cDNA was inserted into pCX [15]. (b) Time schedule for the induction of iPS cells using plasmids. Red arrowheads indicate the timing of transfection. (c) Mouse embryonic fibroblasts (left panel) and induced pluripotent stem cells (right panel).
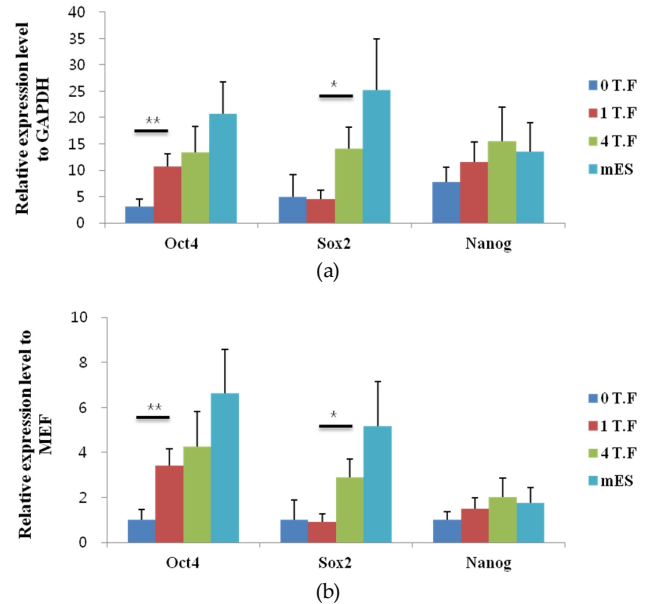

(a)

(b)

Fig. 2. Expression of pluripotency markers. (a) Real-time PCR analysis of pluripotency marker genes in MEFs, 1st-transfected MEFs, 3rd-transfected MEFs, and ES cells. (b) Real-time PCR analysis of pluripotency marker genes in 1st-transfected MEFs, 3rd-transfected MEFs, and ES cells relative to MEFs. Data are presented as the mean±SEM of three independent experiments. * *p* <0.05, ** *p*<0.01, *** *p*<0.001 (Student's *t*-test).

Here, we have taken advantage of a strategy for generating virus-free, factor-removable induced pluripotent stem cells using a single plasmid with a 2A-peptide-linked reprogramming cassette, Oct4-Klf4-Sox2 pCX-OKS-2A. In addition, we purchased another plasmid to express c-Myc (pCX-M-2A) (Addgene) (Fig. 1a). We transfected pCX-OKS-2A and pCX-M-2A into cells on days 2, 6, 10, and 14 (Fig. 2b).

In a study on three-factor reprogramming of human fibroblasts (Nakagawa *et al.*, 2008), infected human cells were first cultured in serum containing fibroblast medium, reseeded on feeders, and then transferred to ES cell medium. Serum replacement, typical of ES cell media, has been shown to increase the efficiency of mouse cell reprogramming (Blelloch *et al.*, 2007). Therefore, changing to mouse ES cell medium with serum replacement sooner might increase the efficiency of mouse fibroblast reprogramming (Huangfu *et al.*, 2008b). Vc, a nutrient vital to human health, enhances the reprogramming of somatic cells to pluripotent stem cells (Esteban *et al.*, 2010). We reasoned that cells were seeded in fibroblast medium immediately after secondary infection and to mouse ES cell medium with serum that contained Vc. Furthermore, DNA methyltransferase and histone deacetylase (HDAC) inhibitors improve reprogramming efficiency. In particular, the HDAC inhibitor VPA improves reprogramming efficiency (Huangfu *et al.*, 2008a; Huangfu *et al.*, 2008b). The effect of

VPA is much stronger than that of other HDAC inhibitors tested, which could be due to the toxicity of other chemicals at higher dosages (Huangfu *et al.*, 2008a). We therefore treated four factor-transfected MEFs with 1 mM VPA for only 1 week (Fig. 2b). MEK inhibition has also been shown to increase the efficiency of the reprogramming of fibroblasts into iPS cells (Lin *et al.*, 2009). Thus, 2 weeks later, transfected cells were incubated with the MEK inhibitor PD0325901 (Stemgent) (Si-Tayeb *et al.*, 2010) (Fig. 2b).

Virus-free ES-cell-like colonies from four factor-transfected mouse fibroblasts can be readily identified by their morphology and picked and expanded to establish iPS cell lines (Fig. 1c).

Virus-Free Induced Pluripotent Stem Cells Express Pluripotency Marker Genes

Oct4 and Sox2 are well known to play important roles in the maintenance of embryonic stem cell pluripotency. These transcription factors bind to regulatory regions within hundreds of target genes to control their expression (Wang *et al.*, 2007). Nanog is a highly divergent homeodomain-containing protein commonly given a central position in the transcriptional network of pluripotency (Boyer *et al.*, 2005; Cole *et al.*, 2008; Loh *et al.*, 2006; Wang *et al.*, 2006). It is expressed in pluripotent embryonic cells, isolated ES cells, and the developing germline in mammals and birds (Chambers *et al.*, 2003; Lavial *et al.*, 2007; Mitsui *et al.*, 2003; Yamaguchi *et al.*, 2005). Forced expression of Nanog is sufficient to drive cytoline-independent self-renewal of undifferentiated ES cells (Chambers *et al.*, 2003).

We measured the mRNA expression of pluripotency marker genes in MEFs, 1st-transfected MEFs, 3rd-transfected MEFs, and ES cells by real-time PCR (Fig. 2a). Expression of pluripotency marker genes in 1st-transfected MEFs, 3rd-transfected MEFs, and ES cells relative to that in MEFs, as assessed by real-time PCR, is shown in Fig. 2b. The results indicate that the expression of pluripotency marker genes in MEFs depend on the number of rounds of transfections.

Characterization of iPS Cells Generated using Nonviral Vectors

We established mouse virus-free ES-cell-like colonies by picking nonviral vector-transfected mouse MEFs within 4~6 weeks after transfection. iPS cells could be expanded under mouse ES cell culture conditions.

Previous studies have shown that cultures used to successfully propagate undifferentiated murine ES cells for many months contain cells that can form single cell-derived colonies of AP-positive cells (Pease, *et al.*, 1990). In addition, the loss of this ability was shown to be one of the earliest indicators of the induction of differentiation (Palmqvist *et al.*, 2005). Cultures used to
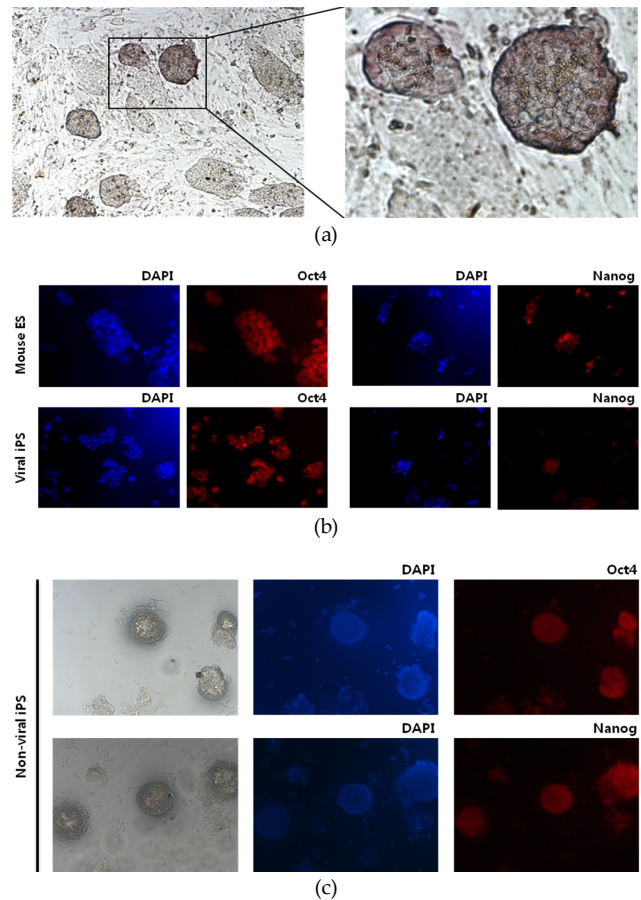

(a)

(b)

(c)

Fig. 3. Characterization of ES, virus iPS, and nonviral iPS cell lines. (a) Nonviral iPS colonies were stained for alkaline phosphatase (AP). (b) Immunocytochemical analysis of pluripotency markers (Oct4, Sox2, and Nanog) in ES, virus iPS, and nonviral iPS cells. Nuclei are stained with DAPI (blue).

successfully propagate human ES cells have also been found to contain cells that produce colonies of AP-positive cells when plated under similar conditions (Xu *et al.*, 2001). iPS cells were positive for AP (Fig. 3a).

Immunofluorescence staining confirmed that iPS cells uniformly expressed mouse ES cell markers, including Oct4 and Nanog (Fig. 3b). These results demonstrate that mouse iPS cells can be generated from MEF cells using nonviral vectors.

In Vitro Differentiation

The ability of ES cells to differentiate into all cell types is the basis of their potential in regenerative medicine, and the formation of EBs is the principal step in the differentiation of ES cells. When cultured in the absence of LIF or MEF feeder layers, ES cells differentiate spontaneously and then form three-dimensional aggregates called EBs. This structure facilitates multicellular interactions in which cell-cell contacts exist and gap junctions may be established. An EB consists of ectodermal, mesodermal, and endodermal tissues, which re-
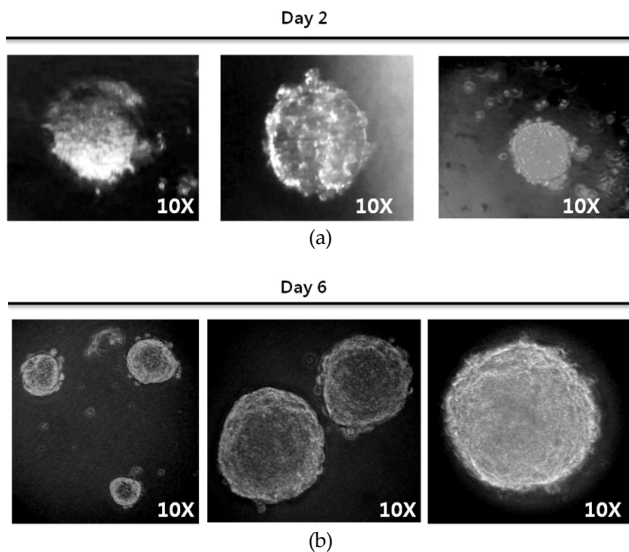
Day 2

(a)

Day 6

(b)

Fig. 4. *In vitro* differentiation of nonviral iPS cells and ES cells. (a) Nonviral iPS cells form embryoid bodies in suspension culture at 2 days. (b) Nonviral iPS cells form embryoid bodies in suspension culture at 6 days.

capitulate many aspects of cell differentiation during early mammalian embryogenesis and differentiate into derivatives of all the three germ layers (Desbaillets *et al.*, 2000; Itskovitz-Eldor *et al.*, 2000). We examined the differentiation capacity of the iPS cells *in vitro*. In the past, researchers allowed EB formation according to a protocol that was designed to satisfy their purposes in a culture method selected from several types. Three basic methods-liquid suspension culture in bacterial-grade dishes, culture in methylcellulose semisolid media, and culture in a hanging drop-are usually used for the formation of EBs to induce the formation of a variety of cell types from ES cells (Hopfl *et al.*, 2004; Keller, 1995). A bacterial-grade dish, which is a non-treated polystyrene dish with hydrophobicity, has been used for liquid suspension culture of ES cells to induce EB formation. In 1985, Doetschman *et al.* (Doetschman *et al.*, 1985) developed a technique for forming EBs from ES cells in suspension culture using bacterial-grade dishes. Irregularly shaped ES-cell aggregates form after 1 day in suspension and develop into spherical EBs by day 2 (Fig. 4a). Figure 4b shows an example of an EB 5 days after aggregation. Note that the aggregate has increased substantially in size as a result of continued proliferation and is, at 6 days, several hundred micrometers in size (Fig. 4b). Like mouse ES cells, nonviral iPS cells form embryoid bodies in suspension culture in bacterial-grade dishes.

DISCUSSION

A somatic cell can be reprogrammed by transferring

its nucleus into an oocyte (Byrne *et al.*, 2007; Gurdon, *et al.*, 1958; Wakayama, *et al.*, 1998; Wilmut, *et al.*, 1997) or by fusion with an ES cell (Cowan *et al.*, 2005; Tada, *et al.*, 2001), indicating that pluripotency can be restored in differentiated cells. Yamanaka and colleagues identified transcription factors that enable the reprogramming of somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006), which are called iPS cells derived from mouse (Maherali *et al.*, 2007; Okita *et al.*, 2007; Wernig, *et al.*, 2007) or human (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007) fibroblasts. However, significant hurdles remain, notably the low efficiency of primary cell reprogramming and the integration of viral transgenes into the somatic genome, especially oncogenes such as c-Myc and Klf4 (Yamanaka, 2009).

In this report, we described the generation of mouse iPS cells devoid of exogenous DNA by the simple transient transfection of a nonviral vector carrying 2A-peptide-linked reprogramming factors. The histone deacetylase inhibitor VPA improves reprogramming efficiency (Huangfu, *et al.*, 2008a), while Vc allows the reprogramming to run more smoothly by facilitating histone demethylation (Esteban *et al.*, 2010). We therefore used VPA, Vc, and the MEK inhibitor PD0325901 to increase the reprogramming efficiency. The reprogrammed somatic cells expressed pluripotency markers and formed EBs. Small-molecule approaches can be used to increase the efficiency of reprogramming. Although the frequency of restoration of completely reprogrammed iPS cells was lower than that achieved through viral infection, the safely of nonviral vector transfection should compensate for the reduced efficiency.

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