Cell

Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors

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SUMMARY

Successful reprogramming of differentiated human somatic cells into a pluripotent state would allow creation of patient- and disease-specific stem cells. We previously reported generation of induced pluripotent stem (iPS) cells, capable of germline transmission, from mouse somatic cells by transduction of four defined transcription factors. Here, we demonstrate the generation of iPS cells from adult human dermal fibroblasts with the same four factors: Oct3/4, Sox2, Klf4, and c-Myc. Human iPS cells were similar to human embryonic stem (ES) cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. Furthermore, these cells could differentiate into cell types of the three germ layers in vitro and in teratomas. These findings demonstrate that iPS cells can be generated from adult human fibroblasts.

INTRODUCTION

Embryonic stem (ES) cells, derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency (Evans and Kaufman, 1981; Martin, 1981). These properties have led to expectations that human ES cells might be useful to understand disease mechanisms, to screen effective and safe drugs, and to treat patients of various diseases and injuries, such as juvenile diabetes and spinal cord injury (Thomson et al., 1998). Use of human embryos, however, faces ethical controversies that hinder the applications of human ES cells. In addition, it is difficult to generate patient- or disease-specific ES cells, which are required for their effective application. One way to circumvent these issues is to induce pluripotent status in somatic cells by direct reprogramming (Yamanaka, 2007).

We showed that induced pluripotent stem (iPS) cells can be generated from mouse embryonic fibroblasts (MEF) and adult mouse tail-tip fibroblasts by the retrovirus-mediated transfection of four transcription factors, namely Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). Mouse iPS cells are indistinguishable from ES cells in morphology, proliferation, gene expression, and teratoma formation. Furthermore, when transplanted into blastocysts, mouse iPS cells can give rise to adult chimeras, which are competent for germline transmission (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). These results are proof of principle that pluripotent stem cells can be generated from somatic cells by the combination of a small number of factors.

In the current study, we sought to generate iPS cells from adult human somatic cells by optimizing retroviral transduction in human fibroblasts and subsequent culture conditions. These efforts have enabled us to generate iPS cells from adult human dermal fibroblasts and other human somatic cells, which are comparable to human ES cells in their differentiation potential in vitro and in teratomas.

RESULTS

Optimization of Retroviral Transduction for Generating Human iPS Cells

Induction of iPS cells from mouse fibroblasts requires retroviruses with high transduction efficiencies (Takahashi and Yamanaka, 2006). We, therefore, optimized transduction methods in adult human dermal fibroblasts (HDF). We first introduced green fluorescent protein (GFP) into adult HDF with amphotropic retrovirus produced in PLAT-A packaging cells. As a control, we introduced GFP to mouse embryonic fibroblasts (MEF) with ecotropic retrovirus produced in PLAT-E packaging cells(Morita et al., 2000). In MEF, more than 80% of cells expressed GFP (Figure S1). In contrast, less than 20% of HDF expressed

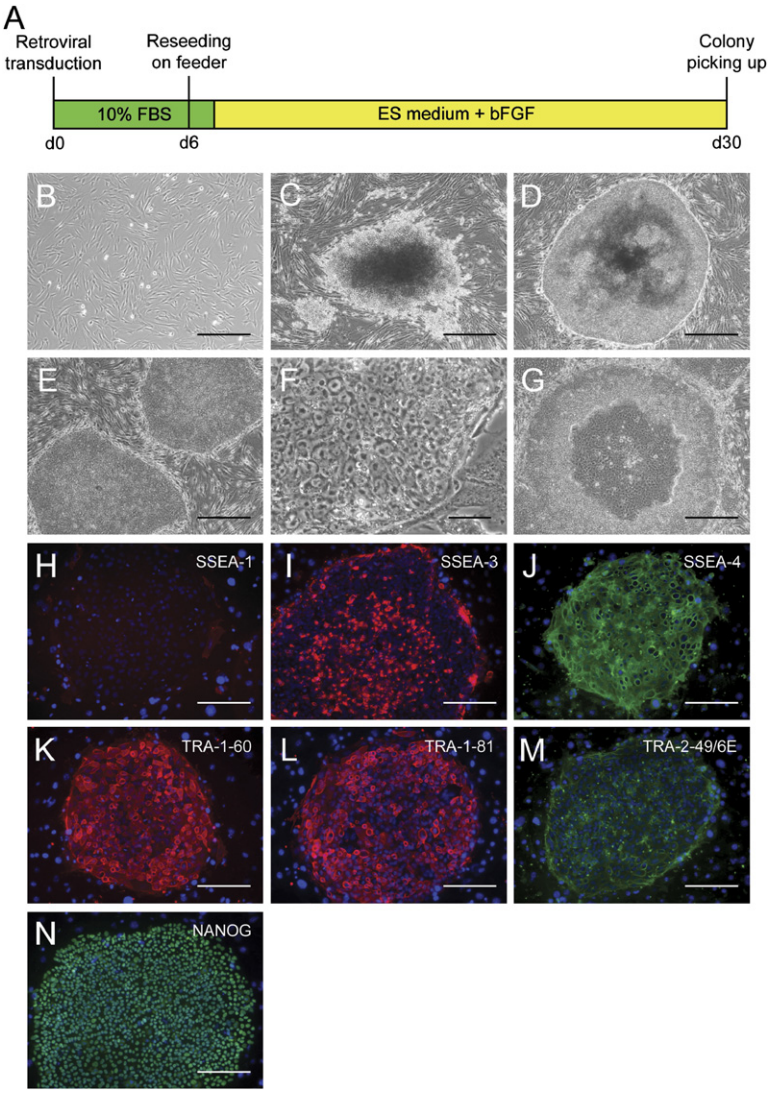

Figure 1. Induction of iPS Cells from Adult HDF

(A) Time schedule of iPS cell generation.
(B) Morphology of HDF.
(C) Typical image of non-ES cell-like colony.
(D) Typical image of hES cell-like colony.
(E) Morphology of established iPS cell line at passage number 6 (clone 201B7).
(F) Image of iPS cells with high magnification.
(G) Spontaneously differentiated cells in the center part of human iPS cell colonies.
(H–N) Immunocytochemistry for SSEA-1 (H), SSEA-3 (I), SSEA-4 (J), TRA-1-60 (K), TRA-1-81 (L), TRA-2-49/6E (M), and Nanog (N). Nuclei were stained with Hoechst 33342 (blue). Bars = 200 μm (B–E, G), 20 μm (F), and 100 μm (H–N).

GFP with significantly lower intensity than in MEF. To improve the transduction efficiency, we introduced the mouse receptor for retroviruses, Slc7a1 (Verrey et al., 2004) (also known as mCAT1), into HDF with lentivirus. We then introduced GFP to HDF-Slc7a1 with ecotropic retrovirus. This strategy yielded a transduction efficiency of 60%, with a similar intensity to that in MEF.

Generation of iPS Cells from Adult HDF

The protocol for human iPS cell induction is summarized in Figure 1A. We introduced the retroviruses containing human Oct3/4, Sox2, Klf4, and c-Myc into HDF-Slc7a1 (Figure 1B; 8×10^5 cells per 100 mm dish). The HDF derived from facial dermis of 36-year-old Caucasian female. Six days after transduction, the cells were harvested by trypsinization and plated onto mitomycin C-treated SNL feeder cells (McMahon and Bradley, 1990) at 5×10^4 or 5×10^5 cells per 100 mm dish. The next day, the medium (DMEM containing 10% FBS) was replaced with a medium for primate ES cell culture supplemented with 4 ng/ml basic fibroblast growth factor (bFGF).

Approximately two weeks later, some granulated colonies appeared that were not similar to hES cells in morphology (Figure 1C). Around day 25, we observed distinct types of colonies that were flat and resembled hES cell colonies (Figure 1D). From 5×10^4 fibroblasts, we observed ~10 hES cell-like colonies and ~100 granulated colonies (7/122, 8/84, 8/171, 5/73, 6/122, and 11/213 in six independent experiments, summarized in Table S1). At day 30, we picked hES cell-like colonies and mechanically disaggregated them into small clumps without enzymatic digestion. When starting with 5×10^5 fibroblasts, the dish was nearly covered with more than 300 granulated colonies. We occasionally observed some hES cell-like colonies in between the granulated cells, but it was difficult to isolate hES cell-like colonies because of the high density of granulated cells. The nature of the non-hES-like cells remains to be determined.

The hES-like cells expanded on SNL feeder cells with the primate ES cell medium containing bFGF. They formed tightly packed and flat colonies (Figure 1E). Each cell exhibited morphology similar to that of human ES cells, characterized by large nuclei and scant cytoplasm (Figure 1F). As is the case with hES cells, we occasionally observed spontaneous differentiation in the center of the colony (Figure 1G).

These cells also showed similarity to hES cells in feeder dependency (Figure S2). They did not attach to gelatin-coated tissue-culture plates. By contrast, they maintained an undifferentiated state on Matrigel-coated plates in MEF-conditioned primate ES cell medium, but not in non-conditioned medium.

Since these cells were similar to hES cells in morphology and other aspects noted above, we will refer to the selected cells after transduction of HDF as human iPS cells, as we describe the molecular and functional evidence for this claim. Human iPS cells clones established in this study are summarized in Table S2.

Human iPS Cells Express hES Markers

In general, except for a few cells at the edge of the colonies, human iPS cells did not express stage-specific embryonic antigen (SSEA)-1 (Figure 1H). In contrast, they expressed hES cell-specific surface antigens(Adewumi et al., 2007), including SSEA-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81 and TRA-2-49/6E (alkaline phosphatase), and NANOG protein (Figures 1I–1N).

RT-PCR showed human iPS cells expressed many undifferentiated ES cell-marker genes (Adewumi et al., 2007), such as *OCT3/4*, *SOX2*, *NANOG*, *growth and differentiation factor 3* (*GDF3*), *reduced expression 1* (*REX1*), *fibroblast growth factor 4* (*FGF4*), *embryonic cell-specific gene 1* (*ESG1*), *developmental pluripotency-associated 2* (*DPPA2*), *DPPA4*, and *telomerase reverse transcriptase* (*hTERT*) at levels equivalent to or higher than those in the hES cell line H9 and the human embryonic carcinoma cell line, NTERA-2 (Figure 2A). By western blotting, proteins levels of OCT3/4, SOX2, NANOG, SALL4, E-CADHERIN, and hTERT were similar in human iPS cells and hES cells (Figure 2B). Although the expression levels of Klf4 and c-Myc increased more than 5-fold in HDF after the retroviral transduction (not shown), their expression levels in human iPS cells were comparable to those in HDF (Figures 2A and 2B), indicating retroviral silencing. RT-PCR using primers specific for retroviral transcripts confirmed efficient silencing of all the four retroviruses (Figure 2C). DNA microarray analyses showed that the global gene-expression patterns are similar, but not identical, between human iPS cells and hES cells (Figure 2D).

Among 32,266 genes analyzed, 5,107 genes showed more than 5-fold difference in expression between HDF and human iPS cells (Tables S3 and S4), whereas 6083 genes between HDF and hES cells showed >5-fold difference in expression (Tables S5 and S6). In contrast, a smaller number of genes (1,267 genes) showed >5-fold difference between human iPS cells and hES cells (Tables S7 and S8).

Promoters of ES Cell-Specific Genes Are Active in Human iPS Cells

Bisulfite genomic sequencing analyses evaluating the methylation statuses of cytosine guanine dinucleotides (CpG) in the promoter regions of pluripotent-associated genes, such as *OCT3/4*, *REX1*, and *NANOG*, revealed that they were highly unmethylated, whereas the CpG dinucleotides of the regions were highly methylated in parental HDFs (Figure 3A). These findings indicate that these promoters are active in human iPS cells.

Luciferase reporter assays also showed that human *OCT3/4* and *REX1* promoters had high levels of transcriptional activity in human iPS cells and EC cells (NTERA-2) but not in HDF. The promoter activities of ubiquitously expressed genes, such as human RNA polymerase II (*PolII*), showed similar activities in both human iPS cells and HDF (Figure 3B).

We also performed chromatin immunoprecipitation to analyze the histone modification status in human iPS cells (Figure 3C). We found that histone H3 lysine 4 was methylated whereas H3 lysine 27 was demethylated in the promoter regions of *Oct3/4*, *Sox2*, and *Nanog* in human iPS cells. We also found that human iPS cells showed the bivalent patterns of development-associated genes, such as *Gata6, Msx2, Pax6,* and *Hand1*. These histone modification statuses are characteristic of hES cells (Pan et al., 2007).

High Telomerase Activity and Exponential Growth of Human iPS Cells

As predicted from the high expression levels of hTERT, human iPS cells showed high telomerase activity (Figure 4A). They proliferated exponentially for as least 4 months (Figure 4B). The calculated population doubling time of human iPS cells were 46.9 ± 12.4 (clone 201B2), 47.8 ± 6.6 (201B6) and 43.2 ± 11.5 (201B7) hours. These times are equivalent to the reported doubling time of hES cells (Cowan et al., 2004).

Embryoid Body-Mediated Differentiation of Human iPS Cells

To determine the differentiation ability of human iPS cells in vitro, we used floating cultivation to form embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000). After 8 days in suspension culture, iPS cells formed ball-shaped structures (Figure 5A). We transferred these embryoid body-like structures to gelatin-coated plates and continued cultivation for another 8 days. Attached cells showed various types of morphologies, such as those resembling neuronal cells, cobblestone-like cells, and epithelial cells (Figures 5B–5E). Immunocytochemistry detected cells positive for βIII-tubulin (a marker of ectoderm), glial fibrillary acidic protein (GFAP, ectoderm), α-smooth muscle actin (α-SMA, mesoderm), desmin (mesoderm), α-fetoprotein (AFP, endoderm), and vimentin (mesoderm and parietal

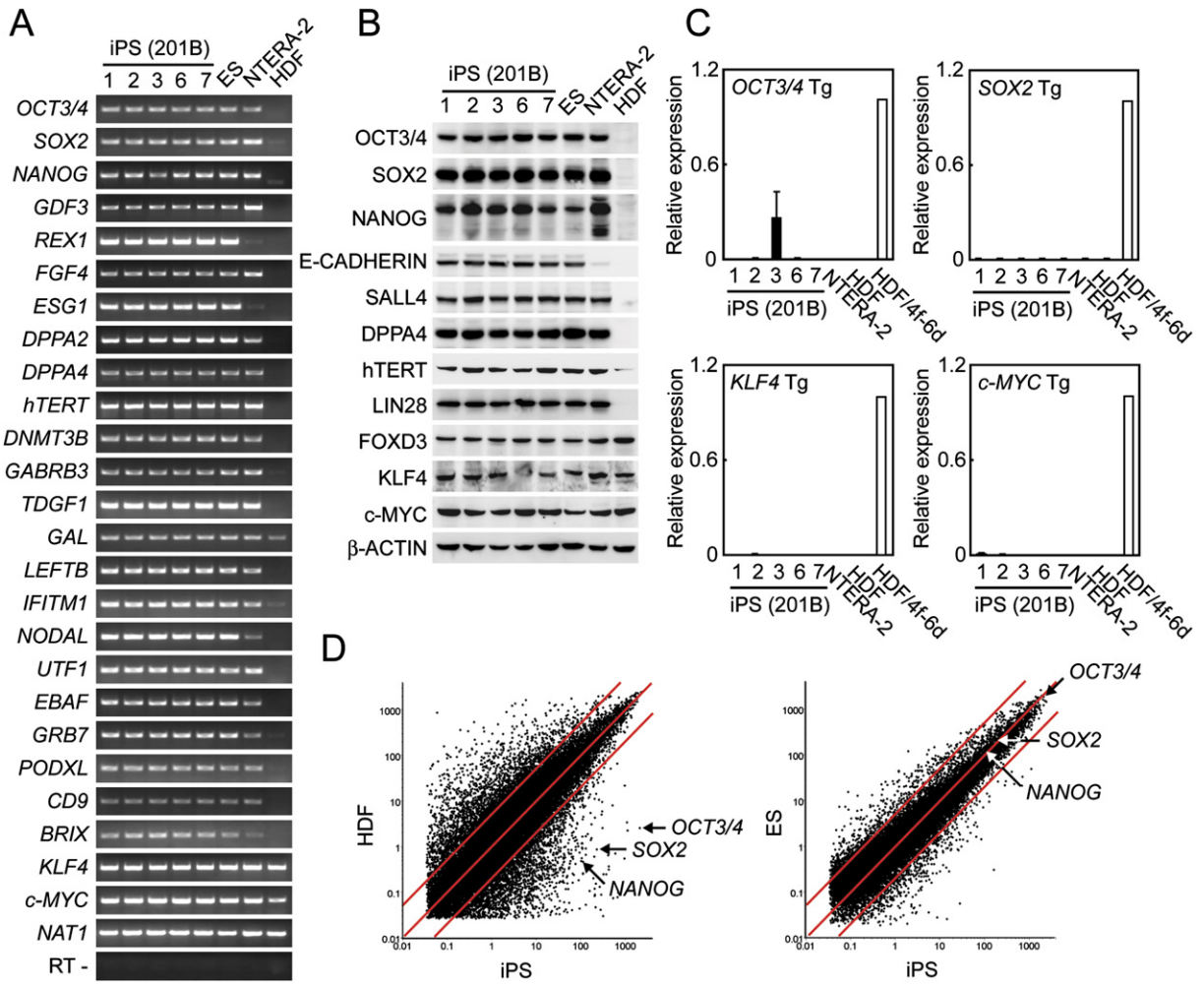

Figure 2. Expression of hES Cell-Marker Genes in Human iPS Cells

(A) RT-PCR analysis of ES cell-marker genes. Primers used for Oct3/4, Sox2, Klf4, and c-Myc specifically detect the transcripts from the endogenous genes, but not from the retroviral transgenes.

(B) Western blot analysis of ES cell-marker genes.

(C) Quantitative PCR for expression of retroviral transgenes in human iPS cells, HDF, and HDF 6 days after the transduction with the four retroviruses (HDF/4f-6d). Shown are the averages and standard deviations of three independent experiments. The value of HDF/4f-6d was set to 1 in each experiment.

(D) The global gene-expression patterns were compared between human iPS cells (clone 201B7) and HDF, and between human iPS cells and hES cells (H9) with oligonucleotide DNA microarrays. Arrows indicate the expression levels of Nanog, endogenous Oct3/4 (the probe derived from the 3′ untranslated region, which does not detect the retroviral transcripts), and endogenous Sox2. The red lines indicate the diagonal and 5-fold changes between the two samples.

endoderm) (Figures 5F–5K). RT-PCR confirmed that these differentiated cells expressed forkhead box A2 (*FOXA2*, a marker of endoderm), AFP (endoderm), cytokeratin 8 and 18 (endoderm), SRY-box containing gene 17 (*SOX17*, endoderm), *BRACHYURY* (mesoderm), Msh homeobox 1 (*MSX1*, mesoderm), microtubule-associated protein 2 (*MAP2*, ectoderm), and paired box 6 (*PAX6*, ectoderm) (Figure 5L). In contrast, expression of *OCT3/4*, *SOX2*, and *NANOG* was markedly decreased. These data demonstrated that iPS cells could differentiate into three germ layers in vitro.

Directed Differentiation of Human iPS Cells into Neural Cells

We next examined whether lineage-directed differentiation of human iPS cells could be induced by reported methods for hES cells. We seeded human iPS cells on PA6 feeder layer and maintained them under differentiation conditions for 2 weeks (Kawasaki et al., 2000). Cells spread drastically, and some neuronal structures were observed (Figure 6A). Immunocytochemistry detected cells positive for tyrosine hydroxylase and βIII tubulin in the culture (Figure 6B). PCR analysis revealed expression of

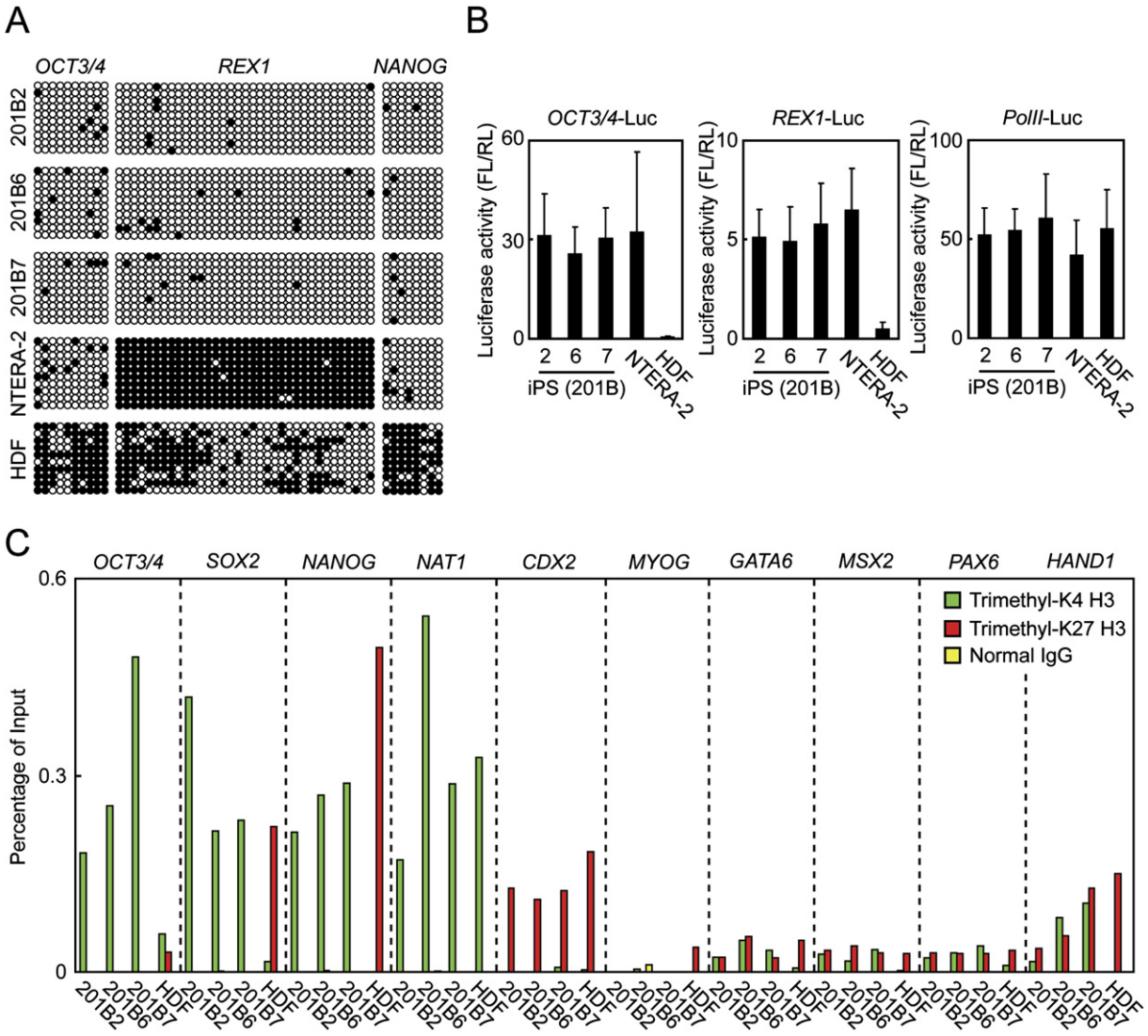

Figure 3. Analyses Promoter Regions of Development-Associated Genes in Human iPS Cells

(A) Bisulfite genomic sequencing of the promoter regions of *OCT3/4*, *REX1*, and *NANOG*. Open and closed circles indicate unmethylated and methylated CpGs.

(B) Luciferase assays. The luciferase reporter construct driven by indicated promoters were introduced into human iPS cells or HDF by lipofection. The graphs show the average of the results from four assays. Bars indicate standard deviation.

(C) Chromatin Immunoprecipitation of histone H3 lysine 4 and lysine 27 methylation.

dopaminergic neuron markers, such as aromatic-L-amino acid decarboxylase (*AADC*), member 3 (*DAT*), choline acetyltransferase (*ChAT*), and LIM homeobox transcription factor 1 beta (*LMX1B*), as well as another neuron marker, *MAP2* (Figure 6C). In contrast, *GFAP* expression was not induced with this system. On the other hand, the expression of *OCT3/4* and *NANOG* decreased markedly, whereas *Sox2* decreased only slightly (Figure 6C). These data demonstrated that iPS cells could differentiate into neuronal cells, including dopaminergic neurons, by coculture with PA6 cells.

Directed Differentiation of Human iPS Cells into Cardiac Cells

We next examined directed cardiac differentiation of human iPS cells with the recently reported protocol, which utilizes activin A and bone morphogenetic protein (BMP) 4 (Laflamme et al., 2007). Twelve days after the induction of differentiation, clumps of cells started beating (Figure 6D and Movie S1). RT-PCR showed that these cells expressed cardiomyocyte markers, such as troponin T type 2 cardiac (*TnTc*); myocyte enhancer factor 2C (*MEF2C*); myosin, light polypeptide 7, regulatory (*MYL2A*); myosin,

revealed that each clone had a unique pattern of retroviral integration sites (Figure S3B). In addition, the patterns of 16 short tandem repeats were completely matched between human iPS clones and parental HDF (Table S9). These patterns differed from any established hES cell lines reported on National Institutes of Health website (http://stemcells.nih.gov/research/nihresearch/scunit/genotyping.htm). In addition, chromosomal G-band analyses showed that human iPS cells had a normal karyotype of 46XX (not shown). Thus, human iPS clones were derived from HDF and were not a result of cross-contamination. Whether generation of human iPS cells depends on minor genetic or epigenetic modification awaits further investigation.

Generation of iPS Cells from Other Human Somatic Cells

In addition to HDF, we used primary human fibroblast-like synoviocytes (HFLS) from synovial tissue of 69-year-old Caucasian male and BJ cells, a cell line established from neonate fibroblasts (Table S1 and S2). From HFLS (5×10^4 cells per 100 mm dish), we obtained more than 600 hundred granulated colonies and 17 hES cell-like colonies (Table S1). We picked six colonies, of which only two were expandable as iPS cells (Figure S4). Dishes seeded with 5×10^5 HFLS were covered with granulated cells, and no hES cell-like colonies were distinguishable. In contrast, we obtained 7 to 8 and ~100 hES cell-like colonies from 5×10^4 and 5×10^5 BJ cells, respectively, with only a few granulated colonies (Table S1). We picked six hES cell-like colonies and generated iPS cells from five colonies (Figure S4). Human iPS cells derived from HFLS and BJ expressed hES cell-marker genes at levels similar to or higher than those in hES cells (Figure S5). They differentiated into all three germ layers through EBs (Figure S6). STR analyses confirmed that iPS-HFLS cells and iPS-BJ cells were derived from HFLS and BJ fibroblasts, respectively (Tables S10 and S11).

DISCUSSION

In this study, we showed that iPS cells can be generated from adult HDF and other somatic cells by retroviral transduction of the same four transcription factors with mouse iPS cells, namely Oct3/4, Sox2, Klf4, and c-Myc. The established human iPS cells are similar to hES cells in many aspects, including morphology, proliferation, feeder dependence, surface markers, gene expression, promoter activities, telomerase activities, in vitro differentiation, and teratoma formation. The four retroviruses are strongly silenced in human iPS cells, indicating that these cells are efficiently reprogrammed and do not depend on continuous expression of the transgenes for self renewal.

hES cells are different from mouse counterparts in many respects (Rao, 2004). hES cell colonies are flatter and do not override each other. hES cells depend on bFGF for self renewal (Amit et al., 2000), whereas mouse ES cells depend on the LIF/Stat3 pathway (Matsuda et al., 1999; Niwa et al., 1998). BMP induces differentiation in hES cells (Xu et al., 2005) but is involved in self renewal of mouse ES cells (Ying et al., 2003). Despite these differences, our data show that the same four transcription factors induce iPS cells in both human and mouse. The four factors, however, could not induce human iPS cells when fibroblasts were kept under the culture condition for mouse ES cells after retroviral transduction (data not shown). These data suggest that the fundamental transcriptional network governing pluripotency is common in human and mice, but extrinsic factors and signals maintaining pluripotency are unique for each species.

Deciphering of the mechanism by which the four factors induce pluripotency in somatic cells remains elusive. The function of Oct3/4 and Sox2 as core transcription factors to determine pluripotency is well documented (Boyer et al., 2005; Loh et al., 2006; Wang et al., 2006). They synergistically upregulate "stemness" genes, while suppressing differentiation-associated genes in both mouse and human ES cells. However, they cannot bind their targets genes in differentiated cells because of other inhibitory mechanisms, including DNA methylation and histone modifications. We speculate that c-Myc and Klf4 modifies chromatin structure so that Oct3/4 and Sox2 can bind to their targets (Yamanaka, 2007). Notably, Klf4 interacts with p300 histone acetyltransferase and regulates gene transcription by modulating histone acetylation (Evans et al., 2007).

The negative role of c-Myc in the self renewal of hES cells was recently reported (Sumi et al., 2007). They showed that forced expression of c-Myc induced differentiation and apoptosis of human ES cells. This is great contrast to the positive role of c-Myc in mouse ES cells (Cartwright et al., 2005). During iPS cell generation, transgenes derived from retroviruses are silenced when the transduced fibroblasts acquire ES-like state. The role of c-Myc in establishing iPS cells may be as a booster of reprogramming rather than a controller of maintenance of pluripotency.

We found that each iPS clone contained three to six retroviral integrations for each factor. Thus, each clone had more than 20 retroviral integration sites in total, which may increase the risk of tumorigenesis. In the case of mouse iPS cells, ~20% of mice derived from iPS cells developed tumors, which were attributable, at least in part, to reactivation of the c-Myc retrovirus (Okita et al., 2007). This issue must be overcome to use iPS cells in human therapies. We have recently found that iPS cells can be generated without Myc retroviruses, albeit with lower efficiency (M. Nakagawa, M. Koyanagi, and S.Y., unpublished data). Nonretroviral methods to introduce the remaining three factors, such as adenoviruses or cell-permeable recombinant proteins, should be examined in future studies. Alternatively, one might be able to identify small molecules that can induce iPS cells, without gene transfer.

As is the case with mouse iPS cells, only a small portion of human fibroblasts that had been transduced with the

four retroviruses acquired iPS cell identity. We obtained ~10 iPS cells colonies from 5 × 10^4 transduced HDF. From a practical point of view, this efficiency is sufficiently high, since multiple iPS cell clones can be obtained from a single experiment. From a scientific point of view, however, the low efficiency raises several possibilities. First, the origin of iPS cells may be undifferentiated stem or progenitor cells coexisting in fibroblast culture. Another possibility is that retroviral integration into some specific loci may be required for iPS cell induction. Finally, minor genetic alterations, which could not be detected by karyotype analyses, or epigenetic alterations are required for iPS cell induction. These issues need to be elucidated in future studies.

Our study has opened an avenue to generate patient- and disease-specific pluripotent stem cells. Even with the presence of retroviral integration, human iPS cells are useful for understanding disease mechanisms, drug screening, and toxicology. For example, hepatocytes derived from iPS cells with various genetic and disease backgrounds can be utilized in predicting liver toxicity of drug candidates. Once the safety issue is overcome, human iPS cells should also be applicable in regenerative medicine. Human iPS cells, however, are not identical to hES cells: DNA microarray analyses detected differences between the two pluripotent stem cell lines. Further studies are essential to determine whether human iPS cells can replace hES in medical applications.

EXPERIMENTAL PROCEDURES

Cell Culture

HDF from facial dermis of 36-year-old Caucasian female and HFLS from synovial tissue of 69-year-old Caucasian male were purchased from Cell Applications, Inc. When received, the population doubling was less than 16 in HDF and 5 in HFLS. We used these cells for the induction of iPS cells within six and four passages after the receipt. BJ fibroblasts from neonatal foreskin and NTERA-2 clone D1 human embryonic carcinoma cells were obtained from American Type Culture Collection. Human fibroblasts, NTERA-2, PLAT-E, and PLAT-A cells were maintained in Dulbecco's modified eagle medium (DMEM, Nacalai Tesque, Japan) containing 10% fetal bovine serum (FBS, Japan Serum) and 0.5% penicillin and streptomycin (Invitrogen). 293FT cells were maintained in DMEM containing 10% FBS, 2 mM L-glutamine (Invitrogen), 1 × 10^{-4} M nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Sigma) and 0.5% penicillin and streptomycin. PA6 stroma cells (RIKEN Bioresource Center, Japan) were maintained in α-MEM containing 10% FBS and 0.5% penicillin and streptomycin. iPS cells were generated and maintained in Primate ES medium (ReproCELL, Japan) supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF, WAKO, Japan). For passaging, human iPS cells were washed once with PBS and then incubated with DMEM/F12 containing 1 mg/ml collagenase IV (Invitrogen) at 37°C. When colonies at the edge of the dish started dissociating from the bottom, DMEF/F12/collangenase was removed and washed with Primate ES cell medium. Cells were scraped and collected into 15 ml conical tube. An appropriate volume of the medium was added, and the contents were transferred to a new dish on SNL feeder cells. The split ratio was routinely 1:3. For feeder-free culture of iPS cells, the plate was coated with 0.3 mg/ml Matrigel (growth-factor reduced, BD Biosciences) at 4°C overnight. The plate was warmed to room temperature before use. Unbound Matrigel was aspirated off and washed out with DMEM/F12. iPS cells were seeded on Matrigel-coated plate in MEF-conditioned or nonconditioned primate ES cell medium, both supplemented with 4 ng/ml bFGF. The medium was changed daily. For preparation of MEF-conditioned medium, MEFs derived from embryonic day 13.5 embryo pool of ICR mice were plated at 1 × 10^6 cells per 100 mm dish and incubated overnight. Next day, the cells were washed once with PBS and cultured in 10 ml of primate ES cell medium. After 24 hr incubation, the supernatant of MEF culture was collected, filtered through a 0.22 μm pore-size filter, and stored at −20°C until use.

Plasmid Construction

The open reading frame of human *OCT3/4* was amplified by RT-PCR and cloned into pCR2.1-TOPO. An EcoRI fragment of pCR2.1-hOCT3/4 was introduced into the EcoRI site of pMXs retroviral vector. To discriminate each experiment, we introduced a 20 bp random sequence, which we designated N_{20} barcode, into the *Not*I/*Sal*I site of *Oct3/4* expression vector. We used a unique barcode sequence in each experiment to avoid interexperimental contamination. The open reading frames of human *SOX2*, *KLF4*, and *c-MYC* were also amplified by RT-PCR and subcloned into pENTR-D-TOPO (Invitrogen). All of the genes subcloned into pENTR-D-TOPO were transferred to pMXs by using the Gateway cloning system (Invitrogen), according to the manufacturer's instructions. Mouse *Slc7a1* ORF was also amplified, subcloned into pENTR-D-TOPO, and transferred to pLenti6/UbC/V5-DEST (Invitrogen) by the Gateway system. The regulatory regions of the human *OCT3/4* gene and the *REX1* gene were amplified by PCR and subcloned into pCRXL-TOPO (Invitrogen). For phOCT4-Luc and phREX1-Luc, the fragments were removed by KpnI/BglII digestion from pCRXL vector and subcloned into the KpnI/BglII site of pGV-BM2. For pPolII-Luc, an *Aat*II (blunted)/*Nhe*I fragment of pQBI-polII was inserted into the KpnI (blunted)/*Nhe*I site of pGV-BM2. All of the fragments were verified by sequencing. Primer sequences are shown in Table S12.

Lentivirus Production and Infection

293FT cells (Invitrogen) were plated at 6 × 10^6 cells per 100 mm dish and incubated overnight. Cells were transfected with 3 μg of pLenti6/UbC-Slc7a1 along with 9 μg of Virapower packaging mix by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after transfection, the supernatant of transfectant was collected and filtered through a 0.45 μm pore-size cellulose acetate filter (Whatman). Human fibroblasts were seeded at 8 × 10^5 cells per 100 mm dish 1 day before transduction. The medium was replaced with virus-containing supernatant supplemented with 4 μg/ml polybrene (Nacalai Tesque), and incubated for 24 hr.

Retroviral Infection and iPS Cell Generation

PLAT-E packaging cells were plated at 8 × 10^6 cells per 100 mm dish and incubated overnight. Next day, the cells were transfected with pMXs vectors with Fugene 6 transfection reagent (Roche). Twenty-four hours after transfection, the medium was collected as the first virus-containing supernatant and replaced with a new medium, which was collected after twenty-four hours as the second virus-containing supernatant. Human fibroblasts expressing mouse *Slc7a1* gene were seeded at 8 × 10^5 cells per 100 mm dish 1 day before transduction. The virus-containing supernatants were filtered through a 0.45 μm pore-size filter and supplemented with 4 μg/ml polybrene. Equal amounts of supernatants containing each of the four retroviruses were mixed, transferred to the fibroblast dish, and incubated overnight. Twenty-four hours after transduction, the virus-containing medium was replaced with the second supernatant. Six days after transduction, fibroblasts were harvested by trypsinization and replated at 5 × 10^4 cells per 100 mm dish on an SNL feeder layer. Next day, the medium was replaced with Primate ES cell medium supplemented with 4 ng/ml bFGF. The medium was changed every other day. Thirty days after transduction, colonies were picked up and transferred into 0.2 ml of Primate ES

cell medium. The colonies were mechanically dissociated to small clamps by pipeting up and down. The cell suspension was transferred on SNL feeder in 24-well plates. We defined this stage as passage 1.

RNA Isolation and Reverse Transcription

Total RNA was purified with Trizol reagent (Invitrogen) and treated with Turbo DNA-free kit (Ambion) to remove genomic DNA contamination. One microgram of total RNA was used for reverse transcription reaction with ReverTraAce-α (Toyobo, Japan) and dT_{20} primer, according to the manufacturer's instructions. PCR was performed with ExTaq (Takara, Japan). Quantitative PCR was performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen) and analyzed with the 7300 real-time PCR system (Applied Biosystems). Primer sequences are shown in Table S12.

Alkaline Phosphatase Staining and Immunocytochemistry

Alkaline phosphatase staining was performed using the Leukocyte Alkaline Phosphatase kit (Sigma). For immunocytochemistry, cells were fixed with PBS containing 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were treated with PBS containing 5% normal goat or donkey serum (Chemicon), 1% bovine serum albumin (BSA, Nacalai tesque), and 0.1% Triton X-100 for 45 min at room temperature. Primary antibodies included SSEA1 (1:100, Developmental Studies Hybridoma Bank), SSEA3 (1:10, a kind gift from Dr. Peter W. Andrews), SSEA4 (1:100, Developmental Studies Hybridoma Bank), TRA-2-49/6E (1:20, Developmental Studies Hybridoma Bank), TRA-1-60 (1:50, a kind gift from Dr. Peter W. Andrews), TRA-1-81 (1:50, a kind gift from Dr. Peter W. Andrews), Nanog (1:20, AF1997, R&D Systems), βIII-tubulin (1:100, CB412, Chemicon), glial fibrillary acidic protein (1:500, Z0334, DAKO), α-smooth muscle actin (pre-diluted, N1584, DAKO), desmin (1:100, RB-9014, Lab Vision), vimentin (1:100, SC-6260, Santa Cruz), α-fetoprotein (1:100, MAB1368, R&D Systems), tyrosine hydroxylase (1:100, AB152, Chemicon). Secondary antibodies used were cyanine 3 (Cy3) -conjugated goat anti-rat IgM (1:500, Jackson Immunoresearch), Alexa546-conjugated goat anti-mouse IgM (1:500, Invitrogen), Alexa488-conjugated goat anti-rabbit IgG (1:500, Invitrogen), Alexa488-conjugated donkey anti-goat IgG (1:500, Invitrogen), Cy3-conjugated goat anti-mouse IgG (1:500, Chemicon), and Alexa488-conjugated goat anti-mouse IgG (1:500, Invitrogen). Nucleuses were stained with 1 μg/ml Hoechst 33342 (Invitrogen).

In Vitro Differentiation

For EB formation, human iPS cells were harvested by treating with collagenase IV. The clumps of the cells were transferred to poly (2-hydroxyrthyl methacrylate)-coated dish in DMEM/F12 containing 20% knockout serum replacement (KSR, Invitrogen), 2 mM L-glutamine, 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol (Invitrogen), and 0.5% penicillin and streptomycin. The medium was changed every other day. After 8 days as a floating culture, EBs were transferred to gelatin-coated plate and cultured in the same medium for another 8 days. Coculture with PA6 was used for differentiation into dopaminergic neurons. PA6 cells were plated on gelatin-coated 6-well plates and incubated for 4 days to reach confluence. Small clumps of iPS cells were plated on PA6-feeder layer in Glasgow minimum essential medium (Invitrogen) containing 10% KSR (Invitrogen), 1×10^{-4} M nonessential amino acids, 1×10^{-4} M 2-mercaptoethanol (Invitrogen), and 0.5% penicillin and streptomycin. For cardiomyocyte differentiation, iPS cells were maintained on Matrigel-coated plate in MEF-CM supplemented with 4 ng/ml bFGF for 6 days. The medium was then replaced with RPMI1640 (Invitrogen) plus B27 supplement (Invitrogen) medium (RPMI/B27), supplemented with 100 ng/ml human recombinant activin A (R & D Systems) for 24 hr, followed by 10 ng/ml human recombinant bone morphologenic protein 4 (BMP4, R&D Systems) for 4 days. After cytokine stimulation, the cells were maintained in RPMI/B27 without any cytokines. The medium was changed every other day.

Bisulfite Sequencing

Genomic DNA (1 μg) was treated with CpGenome DNA modification kit (Chemicon), according to the manufacturer's recommendations. Treated DNA was purified with QIAquick column (QIAGEN). The promoter regions of the human Oct3/4, Nanog, and Rex1 genes were amplified by PCR. The PCR products were subcloned into pCR2.1-TOPO. Ten clones of each sample were verified by sequencing with the M13 universal primer. Primer sequences used for PCR amplification were provided in Table S12.

Luciferase Assay

Each reporter plasmid (1 μg) containing the firefly luciferase gene was introduced into human iPS cells or HDF with 50 ng of pRL-TK (Promega). Forty-eight hours after transfection, the cells were lysed with 1X passive lysis buffer (Promega) and incubated for 15 min at room temperature. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega) and Centro LB 960 detection system (BERTHOLD), according to the manufacturer's protocol.

Teratoma Formation

The cells were harvested by collagenase IV treatment, collected into tubes, and centrifuged, and the pellets were suspended in DMEM/F12. One quarter of the cells from a confluent 100 mm dish was injected subcutaneously to dorsal flank of a SCID mouse (CREA, Japan). Nine weeks after injection, tumors were dissected, weighted, and fixed with PBS containing 4% paraformaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin.

Western Blotting

The cells at semiconfluent state were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, and 0.1% SDS), supplemented with protease inhibitor cocktail (Roche). The cell lysate of MEL-1 hES cell line was purchased from Abcam. Cell lysates (20 μg) were separated by electrophoresis on 8% or 12% SDS-polyacrylamide gel and transferred to a polyvinylidine difluoride membrane (Millipore). The blot was blocked with TBST (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) containing 1% skim milk and then incubated with primary antibody solution at 4°C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr at room temperature. Signals were detected with Immobilon Western chemiluminescent HRP substrate (Millipore) and LAS3000 imaging system (FUJIFILM, Japan). Antibodies used for western blotting were anti-Oct3/4 (1:600, SC-5279, Santa Cruz), anti-Sox2 (1:2000, AB5603, Chemicon), anti-Nanog (1:200, R&D Systems), anti-Klf4 (1:200, SC-20691, Santa Cruz), anti-c-Myc (1:200, SC-764, Santa Cruz), anti-E-cadherin (1:1000, 610182, BD Biosciences), anti-Dppa4 (1:500, ab31648, Abcam), anti-FoxD3 (1:200, AB5687, Chemicon), anti-telomerase (1:1000, ab23699, Abcam), anti-Sall4 (1:400, ab29112, Abcam), anti-LIN-28 (1:500, AF3757, R&D systems), anti-β-actin (1:5000, A5441, Sigma), anti-mouse IgG-HRP (1:3000, #7076, Cell Signaling), anti-rabbit IgG-HRP (1:2000, #7074, Cell Signaling), and anti-goat IgG-HRP (1:3000, SC-2056, Santa Cruz).

Southern Blotting

Genomic DNA (5 μg) was digested with BglII, EcoRI, and *Nco*I overnight. Digested DNA fragments were separated on 0.8% agarose gel and transferred to a nylon membrane (Amersham). The membrane was incubated with digoxigenin (DIG)-labeled DNA probe in DIG Easy Hyb buffer (Roche) at 42°C overnight with constant agitation. After washing, alkaline phosphatase-conjugated anti-DIG antibody (1:10,000, Roche) was added to a membrane. Signals were raised by CDP-star (Roche) and detected by LAS3000 imaging system.

Short Tandem Repeat Analysis and Karyotyping

The genomic DNA was used for PCR with Powerplex 16 system (Promega) and analyzed by ABI PRISM 3100 Genetic analyzer and Gene

Mapper v3.5 (Applied Biosystems). Chromosomal G-band analyses were performed at the Nihon Gene Research Laboratories, Japan.

Detection of Telomerase Activity

Telomerase activity was detected with a TRAPEZE telomerase detection kit (Chemicon), according to the manufacturer's instructions. The samples were separated by TBE-based 10% acrylamide nondenaturing gel electrophoresis. The gel was stained with SYBR Gold (1:10,000, Invitrogen).

Chromatin immunoprecuipitation Assay

Approximately 1 × 10^7 cells were crosslinked with 1% formaldehyde for 5 min at room temperature and quenched by addition of glycine. The cell lysate was sonicated to share chromatin-DNA complex. Immunoprecipitation was performed with Dynabeads Protein G (Invitrogen) -linked anti-trimethyl Lys 4 histone H3 (07-473, Upstate), anti-trimethyl Lys 27 histone H3 (07-449, Upstate) or normal rabbit IgG antibody. Eluates were used for quantitative PCR as templates.

DNA Microarray

Total RNA from HDF and hiPS cells (clone 201B) was labeled with Cy3. Samples were hybridized with Whole Human Genome Microarray 4 × 44K (G4112F, Agilent). Each sample was hybridized once with the one color protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data analyzed by using GeneSpring GX7.3.1 software (Agilent). Two normalization procedures were applied; first, signal intensities less than 0.01 were set to 0.01. Then each chip was normalized to the 50th percentile of the measurements taken from that chip. The microarray data of hES H9 cells (Tesar et al., 2007) were retrieved from GEO DataSets (GSM194390, http://www.ncbi.nlm.nih.gov/sites/entrez?db=gds&cmd=search&term=GSE7902). Genes with "present" flag value in all three samples were used for analyses (32,266 genes). We have deposited the microarray data of HDF and hiPS cells to GEO DataSets with the accession number GSE9561.

Supplemental Data

Supplemental Data include 6 figures, 12 tables, and 1 movie and can be found with this article online at http://www.cell.com/cgi/content/full/131/5/861/DC1/.

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