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Improved Efficiency and Pace of Generating Induced Pluripotent Stem Cells from Human Adult and Fetal Fibroblasts

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ABSTRACT

It was reported recently that human fibroblasts can be reprogrammed into a pluripotent state that resembles that of human embryonic stem (hES) cells. This was achieved by ectopic expression of four genes followed by culture on mouse embryonic fibroblast (MEF) feeders under a condition favoring hES cell growth. However, the efficiency of generating human induced pluripotent stem (iPS) cells is low, especially for postnatal human fibroblasts. We started supplementing with an additional gene or bioactive molecules to increase the efficiency of generating iPS cells from human adult as well as fetal fibroblasts. We report here that adding SV40 large T antigen (T) to either set of the four reprogramming genes previously used enhanced the efficiency by 23–70-fold from both human adult and fetal fibroblasts. Discernible hES-like colonies also emerged 1–2 weeks earlier if T was added. With the improved efficiency, we succeeded in replacing MEFs with immortalized human feeder cells that we previously established for optimal hES cell growth. We further characterized individually picked hES-like colonies after expansion (up to 24 passages). The majority of them expressed various undifferentiated hES markers. Some but not all the hES-like clones can be induced to differentiate into the derivatives of the three embryonic germ layers in both teratoma formation and embryoid body (EB) formation assays. These pluripotent clones also differentiated into trophoblasts after EB formation or bone morphogenetic protein 4 induction as classic hES cells. Using this improved approach, we also generated hES-like cells from homozygous fibroblasts containing the sickle cell anemia mutation Hemoglobin Sickle. STEM CELLS *2008;26: 1998–2005*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

It was first reported in November 2007 that human fibroblasts can be transformed into a pluripotent state that resembles that of human embryonic stem (hES) cells [1, 2]. These induced pluripotent stem (iPS) cells were generated by ectopic expression of four transcription factors. While Takahashi et al. [1] used OCT4, SOX2, c-MYC, and KLF4 (SY4) to reprogram both human and mouse fibroblasts to iPS cells, Yu et al. [2] used a different set of four transgenes: OCT4, SOX2, NANOG, and LIN28 (JT4). It was reported soon after that the efficiency of reprogramming postnatal human fibroblasts is much lower than that of fetal or hES-derived fibroblasts, and additional genes are required to supplement the SY4 combination for efficient reprogramming [3]. Retroviral vectors expressing cDNA encoding the TERT and SV40 large T antigen (T) were supplemented to the SY4 vector cocktail, which led to a significant increase of iPS production from embryonic or fetal fibroblasts. By adding the two additional genes that were previously shown to promote human cell transformation, Park et al. managed to generate iPS-like colonies at the level of 3–21 per 10^5 postnatal human fibroblasts [3]. Subsequently, Lowry et al. [4] reported that by adding NANOG to the SY4 combination, they were able to generate hES-like clones from human neonatal dermal fibroblasts. The complete analysis of pluripotency of such hES-like cells derived from postnatal human fibroblasts, including the in vivo teratoma formation assay, remains to be demonstrated [3, 4].

We report here that adding T to either set of the four reprogramming genes (SY4 or JT4) enhanced the efficiency by 23–70-fold on iPS cell production from both human adult and fetal fibroblasts. Discernible hES-like colonies also emerged

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1–2 weeks earlier if T was added. With improved efficiency, we succeeded in replacing mouse embryonic fibroblast (MEF) feeder cells with immortalized human feeder cells (W3R) that we previously established for optimal hES cell growth [5]. We further characterized individually picked hES-like colonies after expansion (up to 24 passages). The majority of them expressed various undifferentiated hES markers. Some but not all the iPS clones differentiated into the derivatives of the three embryonic germ layers in both teratoma formation and embryoid body (EB) formation assays. These pluripotent clones also differentiated into trophoblasts after EB formation or bone morphogenetic protein 4 (BMP4) induction as classic hES cells. We also generated hES-like cells from homozygous fibroblasts containing the sickle cell anemia mutation.

EXPERIMENTAL PROCEDURES

Transgene Expression from Excisable Lentiviral Vectors

Coding regions of human *OCT4*, *SOX2*, *NANOG*, and *LIN28* genes (from hES cells) were cloned by polymerase chain reaction (PCR) and inserted into the pCR2.1-Topo vector (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The SV40 large T antigen cDNA was amplified from pSG5-Large T (plasmid 9053; Addgene, Cambridge, MA, http://www.addgene.org). After confirmation by DNA sequencing, the coding region of each gene was cloned to a derivative of the improved lentiviral vector backbone that we previously constructed [6]. The PGK.hygro cassette was deleted from the dual, excisable transgene (duet) vector, but the LoxP site and the internal EF1α promoter remained. Each cloned transgene is controlled by the EF1α promoter for its expression, as described by Yu et al. [2].

We also used the previously published lentiviral vectors that should also allow LoxP-mediated excision of the transgene [7] and are available from Addgene. The vectors expressing the coding sequence of mouse Oct4 (plasmid 15952), Sox2 (plasmid 15953), Klf4 (plasmid 15950), and n-Myc (plasmid 15951) were used [7]. These mouse transcription factors are highly homologous to their human counterparts, with identity of 90%, 99%, 94%, and 92%, respectively. The n-Myc gene is structurally and functionally highly related to c-Myc, which was used by several groups, although it is reported that a Myc transgene is not essential for iPS cell production from mouse or human fibroblasts [8, 9].

Recombinant lentiviral viruses were produced as previously described [6, 10], by transfection of 293T cells (CRL-11268; American Type Culture Collection, Manassas, VA, http://www.atcc.org) with a mixture of three plasmids: the transducing vector, a vector expressing the VSV-G envelope protein, and a helper plasmid expressing the HIV-Gag/Pol gene. We also made nonintegrating lentiviral (NIL) viruses by the same procedure except that the third plasmid was different. A vector (a gift from Dr. Donald Kohn) expressing a mutated version of the HIV-Gag/Pol (defective in the integrase activity) was used to replace the wild-type [11]. The measurement of titers (enzyme-linked immunosorbent assay for HIV p24) and use of lentiviral vectors were previously described [6, 10]. Polybrene (6 μg/ml) was used as an attachment factor for transducing the following fibroblasts.

Sources and Culture Conditions of Human Fibroblasts Cells

The three human fibroblast lines used for this study were IMR90 (fetal lung fibroblasts; CCL-186; American Type Culture Collection), 1087sk (adult skin fibroblasts; CCD-2104; American Type Culture Collection), and GM02340 (fetal fibroblasts with the homozygous sickle cell anemia mutation hemoglobin sickle [HbS], purchased from Coriell [Camden, NJ, http://www.coriell.org]). They were all cultured in minimal essential medium with Earle's salts (catalog no. 11095098; Invitrogen) and supplemented with 10% (or 15% for the GM02340 fibroblasts) fetal bovine serum (FBS; Invitrogen or other sources). After gene transduction, cells to be reprogrammed were cultured as hES cells as described below.

Culture Media and Conditions for Expanding Human Embryonic Stem Cells and iPS-Like Cells

Human embryonic stem (ES) cells such as H9 were obtained from WiCell Research Institute (Madison, WI, http://www.wicell.org) and propagated as previously described [5, 12–16]. Human ES or iPS cells (after reprogramming) were cultured with the standard hES cell medium (knockout [KO] Dulbecco's modified Eagle's medium, 20% KO Serum Replacement (Invitrogen) with 4 ng/ml basic fibroblast growth factor) on irradiated feeders of either primary MEFs or immortalized human fibroblasts [5, 13, 14, 16]. For the feeder-free culture, undifferentiated hES cell clumps were passaged onto Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com)-coated tissue plates and cultured in pMEF-conditioned medium [13, 14]. The karyotyping was conducted as previously described [12, 14].

Induced hES Cell Differentiation

Various hES cell lines cultured under the feeder-free condition were used for embryoid body formation (in suspension, with 20% FBS) as previously described [14, 15]. For BMP4-induced differentiation, hES cells were cultured under the feeder-free condition (on Matrigel, serum-free, and with pMEF-conditioned medium) in the presence of exogenous BMP4 [16]. Unless otherwise indicated, 50 ng/ml BMP4 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) was used. After 7 days of treatment, the BMP4-treated cells were analyzed by immunostaining [16].

Immunostaining

Adherent hES cells before or after differentiation were fixed with PBS containing 4% paraformaldehyde for 20 minutes at room temperature. Following blocking of nonspecific binding with 4% goat serum, the cells were incubated with mouse or rat monoclonal antibodies. The stained cells were visualized by a secondary IgG conjugated with Alexa 594 or Alexa 555 (Invitrogen). The following antibodies were used in this study: cell antigen, surface Tra-1–60 (MAB4360; Chemicon, Temecula, CA, http://www.chemicon.com) and CD34 (CD34-581; Caltag Laboratories, Burlingame, CA, http://www.caltag.com). Intracellular antigens TROMA-I (Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww), nestin (ab6320-50; Abcam, Cambridge, MA, http://www.abcam.com), human α-protein (N1501; Dako, Glostrup, Denmark, http://www.dako.com), and OCT4 (sc-5299; Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) were used after fixed cells were first permeabilized by treatment with 0.1% Triton X-100 and 1% bovine serum albumin for 45 minutes. Fluorescence-activated cell sorting analysis was described previously [5, 6, 12–16].

Genomic PCR for DNA Fingerprinting

We performed DNA fingerprinting using Invitrogen's MapPairs primers for PCR as described [3]. Genomic DNA was isolated from parental fibroblasts, various hES-like (iPS) cells, and H9 hES cells, using the DNeasy kit (Qiagen, Hilden, Germany, http://www1.qiagen.com). The primer set for the locus D21S2055-R was found to be the best to distinguish the genotypes of various cells used in this study, and the data are shown in Figure 2.

Statistical Analysis

Data plotted are typically expressed as mean $\pm$ SD. Statistical analysis was performed using GraphPad Prism, version 4.0 (GraphPad Software, Inc., San Diego, http://www.graphpad.com). Significance of differences was examined using the Student t test (two-sided, unequal variance) or, when sample sizes were smaller, the nonparametric Mann-Whitney rank sum test. Values were considered significant if $p \leq .05$.

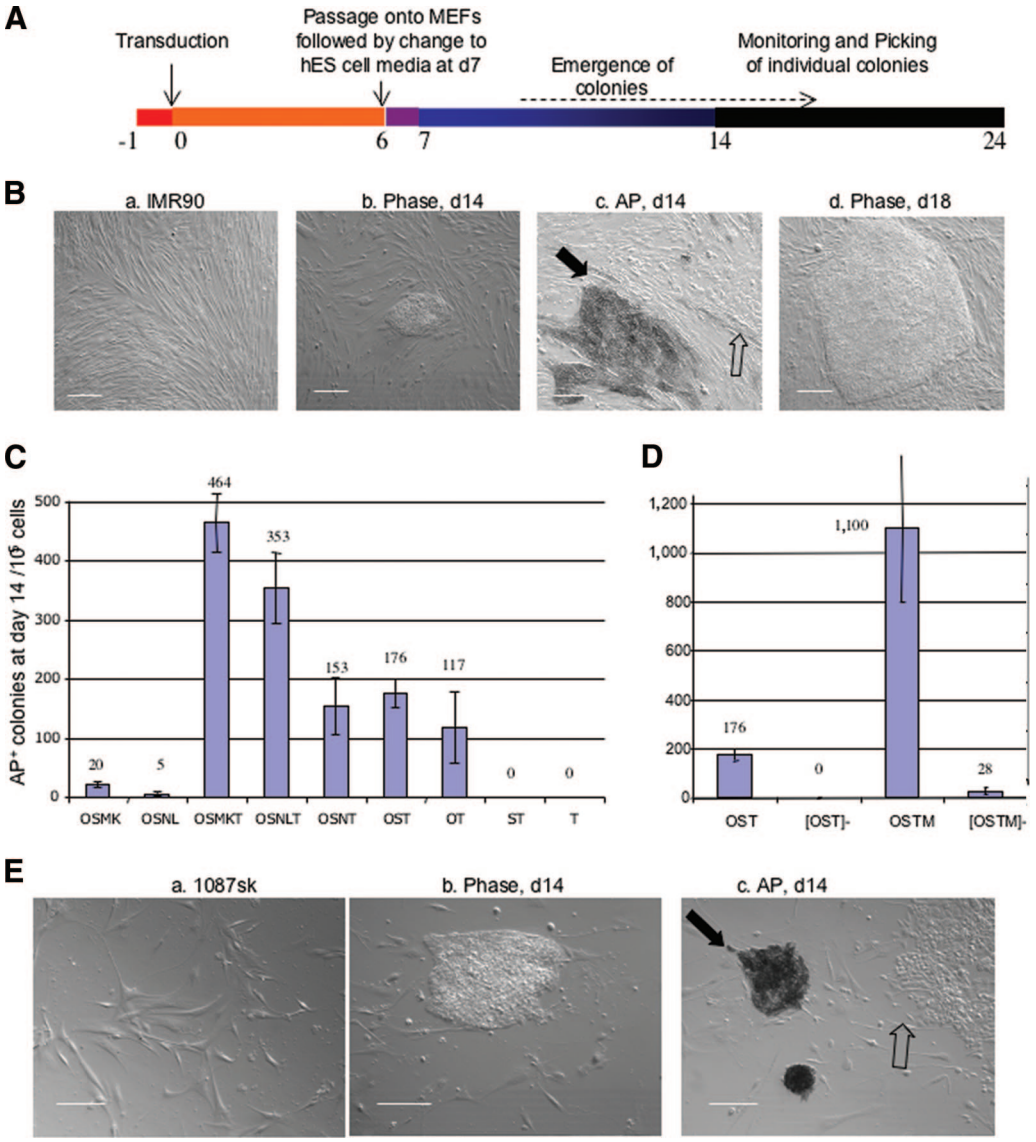

Figure 1. Reprogramming of human adult and fetal fibroblast cells with novel combinations of genes expressed by integrating lentiviral (IL) and nonintegrating lentiviral (NIL) vectors. **(A):** A schematic diagram of the reprogramming protocol used. Human fibroblasts were plated at day −1 and transduced by lentiviral vector cocktails at day 0. Transduced cells were cultured for 6 days in the fetal bovine serum-containing medium used for fibroblasts and then were harvested and counted. The single-cell suspension was then plated onto a preformed MEF monolayer. Cell media were changed next day to the standard hES medium. Emergence of colonies was monitored daily until discernible hES-like colonies were picked (days 14–24). **(B):** Representative images of IMR90 cells (**Ba**) or colonies in situ during reprogramming (**Bb**, **Bc**, and **Bd**). **(C):** Numbers of AP+, hES-like colonies as one shown in (**[Bc]**, filled arrow) observed at day 14 per 10^5 IMR90 cells transduced by various IL vector combinations as indicated. Mean and SD values were from at least two duplicates. Colonies negative for AP (**[Bc]**, open arrow) were also observed but were not counted. Adding T into vector combinations resulted in hES-like colonies at days 8–14 **(Bb, Bc)**. In contrast, these types of colonies did not emerge until days 18–20 by the combinations of OSMK (SY4) or OSNL (JT4), even though few compact colonies containing AP+ cells were observed at day 14. **(D):** Numbers of AP+ colonies at day 14 after transduction of 10^5 IMR90 cells by the combinations of regular IL vectors (as in **[C]**) or NIL vectors made from the same vector plasmid. The combinations of OST with or without M vectors were used. The NIL combinations are denoted as [OST]- and [OSTM]-. Although discernible hES-like colonies were observed as early as day 6 by the IL combination of OSTM, the NIL combination of OSTM ([OSTM]-) did not generate hES-like colonies until days 10–14. **(E):** Morphologies of 1087sk adult human fibroblasts **(A)** and programmed colonies at day 14 in the absence **(B)** and presence **(C)** of fixing and AP staining. Both AP+ (filled arrow) and AP− (open arrow) colonies were observed. Scale bars = 100 μm. Abbreviations: AP, alkaline phosphatase; d, days; hES, human embryonic stem; K, KLF4; L, LIN28; M, MYC; MEF, mouse embryonic fibroblast; N, NANOG; O, OCT4; S, SOX2; T, SV40 large T antigen.

RESULTS

Supplementing a Vector Expressing T Significantly Enhanced the Reprogramming Efficiency from Human Adult and Fetal Fibroblasts

First, we compared side-by-side the efficiency of iPS cell production by transduction of lentiviral vectors expressing either SY4 or JT4 genes from the same human IMR90 fetal fibroblasts used by Yu et al. [2], in the absence or presence of additional genes (Fig. 1). The protocol we took (Fig. 1A) is similar to the report when SY4 was used [1]. Transduced fibroblasts were harvested 6 days after transduction, resuspended as single cells, plated on MEF feeders, and cultured as hES cells with the standard hES medium [11–13]. We confirmed that human fetal IMR90 fibroblasts can be reprogrammed to form hES-like col-

onies ~20 days after transduction by JT4 (Fig. 1C, OSNL). Similar results were also achieved by SY4 (Fig. 1C, OSMK). The SY4 transduction resulted in visible apoptosis and cellular transformation, but also in more and bigger hES-like compact colonies than JT4 by day 14. Colonies that contain alkaline phosphatase (AP)-positive (+) cells at day 14 were found and counted (Fig. 1C).

We next supplemented either the SY4 or the JT4 transgene combination with additional transgenes encoding potent activators of cellular immortalization and transformation [17, 18]. Lentiviral vectors expressing the T or oncogenic H-Ras (G12V) gene were added to each set of four transgenes, which are also delivered by lentiviral vectors [6, 7]. The addition of the oncogenic H-Ras had little effect on the appearance of hES-like colonies from transduced IMR90 fibroblasts in the presence or absence of T (data not shown). However, adding T to either SY4 or JT4 vector cocktail enhanced cell growth and increased the number of hES-like, AP+ colonies by 23-fold over SY4 alone and by 70-fold over JT4 alone (Fig. 1Bc, 1C). The increased efficiency of the AP+ colonies formed was not solely due to the T-enhanced cell growth observed at day 6 (as compared with the input, 1.6× by SY4, 5.2× by SY4+T, 4.2× by JT4, and 9.2× by JT4+T). Distinct hES-like compact colonies also appeared earlier: by 8–10 days after transduction versus 20 days or later when T was omitted. By day 14, ~460 AP+ colonies emerged from 10^5 starting IMR90 cells after SY4+T transduction (Fig. 1C). The colonies generated by JT4+T transduction emerged slightly more slowly and were smaller in size than SY4+T colonies (Fig. 1B). Nonetheless, on average, 353 AP+ colonies were formed of 10^5 starting cells. The great enhancement by T was also observed with adult human skin fibroblasts (1087sk) immortalized by the TERT gene [13, 14]. The combination of SY4+T generated abundantly hES-like colonies stained AP+ by day 14 (50 ± 5 per 10^5 input cells), whereas SY4 alone did not generate any AP+ colonies for this cell type over 24 days we monitored. Our results corroborate those of Park et al. [3] showing that the generation of human iPS cells from both adult and fetal fibroblasts can be improved by using additional genes or optimized combinations. It is possible that adding the TERT gene could further increase the efficiency, especially in telomerase-negative human somatic cells.

We next evaluated whether the T addition would also corroborate parts of the four reprogramming transgenes (Fig. 1C). We found that T potentiated production of AP+, compact, hES-like colonies at day 14 when combined with OCT4/SOX2/NANOG (OSN), OCT4/SOX2 (OS), or OCT4 (O) alone (otherwise no such colonies formed). T alone or with SOX2 (ST) stimulated cell proliferation but did not result in any AP+ compact hES-like colonies by day 14 (Fig. 1C). In a separate series of experiments, we added n-Myc as well as T to the minimal two-gene set *OCT4* and *SOX2*; these genes are shared by both SY4 and JT4 combinations (Fig. 1D). Adding Myc together with T also further significantly enhanced the production of AP+, hES-like colonies (Fig. 1D). The new four-gene combination OCT4/SOX2/T antigen/MYC (OSTM) (essentially adding T to replace KLF4) is 55-fold more efficient over the SY4 (OCT4/SOX2/MYC/KLF4 [OSMK]) combination (Fig. 1C). Similar levels of the reprogramming enhancement by T were also seen with 1087sk adult skin fibroblasts when T was combined with OCT4/SOX2/NANOG/LIN28 (OSNL), OS, or OCT4/SOX2/MYC (OSM) (data not shown). Since the overall reprogramming efficiencies from the human adult fibroblasts were ~12-fold lower than from IMR90 fetal fibroblasts under each gene combination, the addition of the T vector was more critical to obtaining and picking sufficient numbers of hES-like colonies.

Use of NIL Vectors to Achieve Transient Expression

We have attempted to express T and other transgenes transiently by using NIL vectors that are fully functional in infecting cells and reverse transcription to generate DNA forms in transduced cells, but defective in chromosome integration. It was reported that viral DNA from the NIL virus remains episomal for a while and allows transient transgene expression [11]. The level of transgene expression is reduced by one-half along each cell cycle and then is largely diminished after ~1 week in dividing cells. Further analysis reveals that the DNA integration rate of a NIL vector is reduced by 10^4-fold compared with the normal integrating lentiviral (IL) virus [11]. We made both IL and NIL versions of lentiviral viruses from the same plasmid vectors in 293T cells after transfection by using either a wild-type Gag/Pol helper plasmid or a mutated one defective in the integrase activity, respectively. Consistent with previous reports [11], viral titers as measured by lentiviral p24 of the NIL vectors were similar to the integrated version from the same vector. We transduced IMR90 fibroblasts cells by either IL or NIL virus cocktails (Fig. 1D). First, we noted that additional oncogenes (here, Myc) can increase the relative efficiency by at least fivefold and also increase the pace of reprogramming by approximately a week. This enabled us to derive hES cell-like colonies using a cocktail of NIL vectors ([OSTM]−), although it was >50-fold less efficient than by the IL vector counterpart. Moreover, hES-like colonies emerged ~1 week later. Nonetheless, we observed seven AP+, hES-like colonies (from 0.25 × 10^5 IMR90 cells) by using the NIL OSTM combination; all seven colonies were picked, and all were successfully expanded.

Similarly we tested the NIL strategy for human adult skin 1087sk fibroblasts. Since the reprogramming efficiency of 1087sk fibroblasts (even after TERT-mediated immortalization) is much lower than that of IMR90 fetal fibroblasts, we only used the NIL or IL form of the T vector and supplemented the SY4 genes delivered by the standard IL vectors. When the NIL T vector was used, 9 (± 2) AP+, ES-like colonies were observed, compared with 50 (± 5) if the IL T vector was used to transduce 10^5 adult skin 1087sk fibroblasts. This series of experiments was also repeated using 1087sk-derived W3R immortalized fibroblasts as feeders to replace MEFs [5]. Six days after transduction by the NIL T vector and the SY4 IL vectors, transduced 1087sk adult fibroblasts were plated on the "autologous" W3R feeders instead of MEFs (Fig. 1E). We obtained, on average, nine hES-like colonies by day 14 per 10^5 input 1087sk adult fibroblasts (Fig. 1Ec). Five individual colonies were successfully expanded on W3R feeder cells. The W3R human feeders also supported the derivation of human iPS cells derived from IMR90 fibroblasts (not shown).

When we analyzed the presence of the T transgene, we found that all five expanded clones contained the T DNA in the genomic DNA, as in the IL group with the same T vector. Similarly, all seven clones obtained by the NIL OSTM transduction (described above) also contained the T DNA. Considering that the integration rate of NIL vectors is supposed to be 10^4-fold lower, these data strongly suggested that sustained presence of T is likely critical to the enhanced reprogramming of human fibroblasts under the conditions we used.

Initial Characterization of hES-Like Cells Reprogrammed from Fibroblasts

We routinely picked individual compact colonies by days 14–18, which is feasible if T is included in the vector cocktail. Some representative colonies after expansion (up to 24 passages by April 1, 2008) from earlier derivations are shown in Figure 2. Two types of colonies were observed, as shown in Figure 2A. One type, represented by MP2, resembles typical human ES

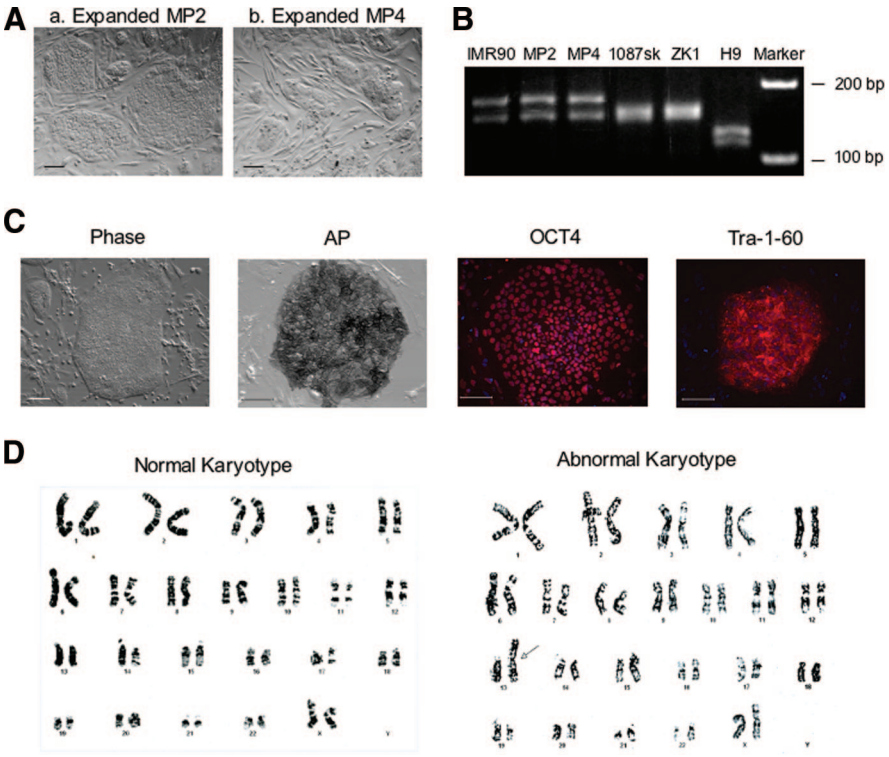

Figure 2. Analysis of human embryonic stem (hES)-like reprogrammed colonies from human fibroblasts. After reprogramming by transgene induction (JT4+T), individually picked colonies (at day 14) were expanded by serial passages under the standard culture for hES cells. After five passages, it was obvious that there were two types of colonies with stable morphologies **(A)**. The majority resembled hES cells, as shown in **(Aa)**, for the expanded MP2 clone. However, we also observed a different type of culture, represented by the expanded MP4 clone **(Ab)**. **(B):** Polymerase chain reaction-based DNA fingerprinting using the MapPairs primers D21S2055-R confirmed that hES-like cells from MP2 and MP4 clones are derived from IMR90 fibroblasts. Also, reprogrammed hES-like cells ZK1 are identical to the parental 1087sk (adult skin) fibroblasts on this DNA locus. The hES-like cells induced from either fetal or adult fibroblasts are distinguishable from H9 hES cells we have in culture. **(C):** We further examined the expression of undifferentiated markers in the reprogrammed cells, such as MP2, which resemble hES cells. MP2 cells expressed AP, Oct4, and Tra-1–60. **(D):** The karyotype of reprogrammed cells, such as MP2, which resemble hES cells, was also examined. Human cell cultures with normal and abnormal karyotype are shown. The arrow points to the abnormality at chromosome 13 (right panel). Abbreviations: AP, alkaline phosphatase; bp, base pairs.

cells (Fig. 2Aa) and expresses undifferentiated markers, such as AP, Oct4, and Tra-1–60 (Fig. 2B). We also observed the existence of colonies such as MP4, which grew like embryonal carcinoma (EC) cells and were independent of the requirements for feeder cells, the MEF-conditioned medium, or fibroblast growth factor. Unlike MP2 cells, MP4 cells expressed undifferentiated markers such as AP and Oct4 but not Tra-1–60 (data not shown).

The colonies from the earliest derivations have been extensively characterized by a variety of techniques, such as DNA fingerprinting, to authenticate the IMR90 origin (Fig. 2C). For hES-like colonies such as MP2, we also performed karyotyping after expansion to obtain sufficient cells. We found that after 10 passages, 10% of MP2 cells displayed the normal 44+XX karyotype, as did the parental IMR90 cells, but the majority of them (18 of 20) displayed an abnormal karyotype (Fig. 2D). After an additional 10 passages, all the MP2 cells carried an abnormal karyotype, consistent with a previously described phenomenon that abnormal cells will progressively take over, probably because of their acquired growth advantages [19]. It remains to be determined how much T or other inserting transgenes contributed to the increased level of an abnormal karyotype observed in this study.

Derivation of hES-Like Cells from Homozygous Fibroblasts Containing the Sickle Cell Anemia Mutation

With an improved efficiency, we next attempted to derive human iPS cells containing a defined mutation associated with a human disease (Fig. 3). We transduced fibroblasts (GM02340) homozygous for the sickle cell anemia mutation HbS (OMIM 603903). Compact hES-like colonies were observed and picked at day 14 (Fig. 3Aa). Three clones were successfully expanded. All of them displayed characteristic hES morphology and undifferentiated markers (Fig. 3Ab, 3Ac). Genomic DNA was isolated, and DNA fingerprinting showed that the clones were derived from the GM02340 fibroblasts and were different from those derived from IMR90, 1087sk, or H9 hES cells (Fig. 3B). DNA of the β-globin locus was amplified and sequenced. As expected, a single point mutation (A to T) was found in codon 6, resulting in the substitution of Glu to Val, characteristic of the HbS mutation (Fig. 3C). That the origin was the MS1 hES-like cells was further confirmed by the presence of a polymorphic (silent) mutation (T to C, without changing codon 2) in both the derived MS1 line and the parental GM02340 fibroblasts. Therefore, we have derived an hES-like cell line in which both alleles of β-globin are mutated to HbS.

In Vitro and In Vivo Pluripotency Tests of Reprogrammed Human Fibroblasts

Upon differentiation induction by forming EBs in suspension or BMP4 as monolayer cells, MP2 generated a variety of cells expressing markers for ectoderm, mesoderm, endoderm, and trophectoderm (Fig. 4A). In contrast, MP4 was unable to generate any overtly differentiated cells under the same conditions used for MP2 (data not shown). Differentiation potential of MP2 and MP4 clones was tested in vivo by the teratoma assay using immunodeficient mice. Both clones formed tumors. When sections of teratomas were examined histologically, many differentiated cell types derived from all the three embryonic lineages can be easily found in the MP2 tumors, but not in any of the seven slides covering various regions of the whole tumor from the MP4 cells. Therefore, we observed two types of reprogrammed cells derived and cultured under the same condition. We observed that hES-like cells, such as MP2, are truly pluripotent as classic hES cells and are qualified to be called human iPS cells. However, we also obtained clones, such as MP4, that resemble undifferentiated ES or EC cells but fail to differentiate

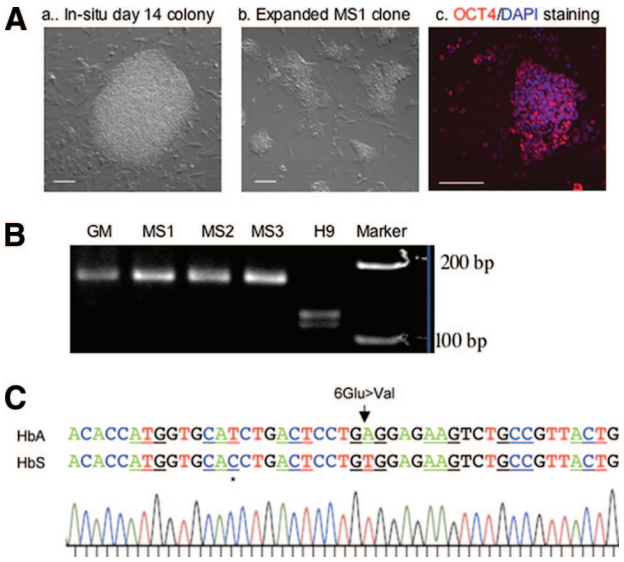

Figure 3. Generation of human embryonic stem (hES)-like cells from fibroblasts containing the homozygous HbS mutation. **(A):** Using an improved method, hES-like colonies emerged at days 10–14 after transduction of four transgenes **(Aa)**. Individual colonies were picked and expanded under the culture condition for hES cells, and one MS1 is shown **(Ab)**. MS1 colonies resemble hES cells and expressed hES cell markers such as OCT4 **(Ac)**. Scale bars = 100 μm. **(B):** Polymerase chain reaction-based DNA fingerprinting confirmed that MS1, MS2, and MS3 are identical to the parental GM but different from H9 hES cells and IMR90-derived induced pluripotent stem cells. **(C):** DNA sequencing data of the MS1 clone. The DNA sequence is matched in color with the original trace. The coding sequence triplets were marked alternately by underline starting from start codon ATG. The wild-type β subunit of HbA sequence is shown at the top. The DNA sequence from the MS1 clone is shown at the bottom. Identical to the original fibroblasts, it contains the HbS mutation at codon 6 (GAG to GTG), resulting in a Glu to Val substitution. The MS1 clone also has a polymorphic silent change (*) as in the original fibroblasts. A single identity of DNA sequence (of multiple clones and both strands) indicates that MS1 is homozygous for the HbS mutation. Abbreviations: bp, base pairs; DAPI, 4,6-diamidino-2-phenylindole; GM, GM02340 fibroblasts; HbA, adult hemoglobin; HbS, hemoglobin sickle.

as observed with some human EC cells with impaired differentiation potential (nullipotent).

DISCUSSION

To understand and improve reprogramming of human fibroblasts to an hES-like state by defined factors, we tested the effects of supplementing and substituting with different genes. We observed that adding T to either the SY4 or the JT4 combination increased efficiency of human iPS cell production by 23–70-fold. In addition, T also enhanced the iPS production efficiency and pace when NANOG, KLF4, LIN28, or MYC was omitted. Therefore, the effect of T is not a simple substitution of functions by one of these factors. When combined with OCT4 and SOX2 (two factors that are necessary but not sufficient to reprogram fibroblasts), T significantly improved the efficiency and pace of generating hES-like colonies. Large, compact, AP+ hES-like colonies were observed at day 12 versus days 20–30, as reported previously when the SY4 or JY4 combination was used [1, 2].

The SV40-encoded 708-amino acid Large T antigen, which contains multiple domains and exerts multiple functions, has been intensively studied in the past three decades and continues to contribute to our understanding of tumor transformation and other biological progresses [20]. In addition to being an essential protein for SV40 DNA replication [21, 22], T simultaneously disables the retinoblastoma and p53 tumor suppressor pathways and alters other cellular targets [20, 23–25]. It is now widely believed that T is not sufficient to transform normal human cells, such as fibroblasts, even in the presence of other factors, such as hTERT, oncogenic Ras, or Myc [17, 18, 20, 23–25]. The SV40 viral transforming activity is in part due to the 172-amino acid small t antigen that is also coded by the SV40 early region. These findings indicate that the minimum number of events required for malignant transformation of human fibroblasts is greater than has been enumerated by such oncogene addition strategies. It is of great interest to determine whether perturbations of cellular targets by T observed in cellular transformation are also critical in the T-mediated enhancement of iPS cell production.

It did not escape our attention that T is also present in the only human cell type so far that had been successfully programmed by protein extracts from human EC cells or mouse ES cells [26, 27]. Human 293T cells were used as recipient (target) cells, which are derived from adenovirus-transformed human embryonic kidney 293 cells by stable transfection with a SV40 T-expressing vector. Permeabilized 293T cells were treated for 1 hour with an extract of undifferentiated human NCCIT EC cells, mouse ES cells, or control cell types such as Jurkat. Amazingly, it was reported that some of the surviving 293T cells after the treatment stably transit to a pluripotent cell phenotype using human EC or mouse ES cell extracts [26, 27]. Clones of primed 293T cells after ≥24 passages expressed hundreds of EC/ES-specific genes, such as *OCT4* and *NANOG*, concomitant with downregulation of 293T-specific genes and of indicators of differentiation markers [26, 27]. Although the source of protein extracts is critical (Jurkat and other somatic cells are insufficient), these reports did not show the evidence of somatic cell priming or reprogramming from any human cell type where T is absent. It is interesting to determine how much the presence of T in 293T cells contributed to the observed programming by protein extracts and whether loading the purified T protein would enhance the programming of cell types free of T.

It is highly desirable that we obtain human iPS cells with high efficiencies without the integration of vectors expressing oncogenic proteins such as MYC and T. We initially focused on viral-mediated systems, which are currently the most efficient way to deliver transgene expression. We chose NIL to deliver either T only (along with other required genes by the standard lentiviral vectors) or all four genes sufficient for reprogramming (Fig. 1D). Human iPS cells were generated and expanded, albeit at a lower efficiency and slower pace compared with the condition using all integrating vectors. However, we detected the presence of T in the genomic DNA in 12 of 12 iPS-like clones we generated using an NIL T vector. This frequency is much higher than predicted (nonspecific) integration of NIL episomal DNA, which was reported to be 10^{-4} compared with IL vectors [11]. These data indirectly but strongly suggest that sustained presence of T is likely critical to the enhanced reprogramming of human fibroblasts under the conditions we used. Since episomal DNA of recombinant adenoviral and adenovirus-associated viral vectors also integrate into the cellular genome at a frequency of 10^{-3} to 10^{-5} per infected cell [28, 29], we predict that the vector integration is also a favorable event if the transgene prolonged expression offers a selective advantage. This information should help to devise strategies to provide prolonged persistence of reprogramming factors without DNA integration of T and other gene vectors.

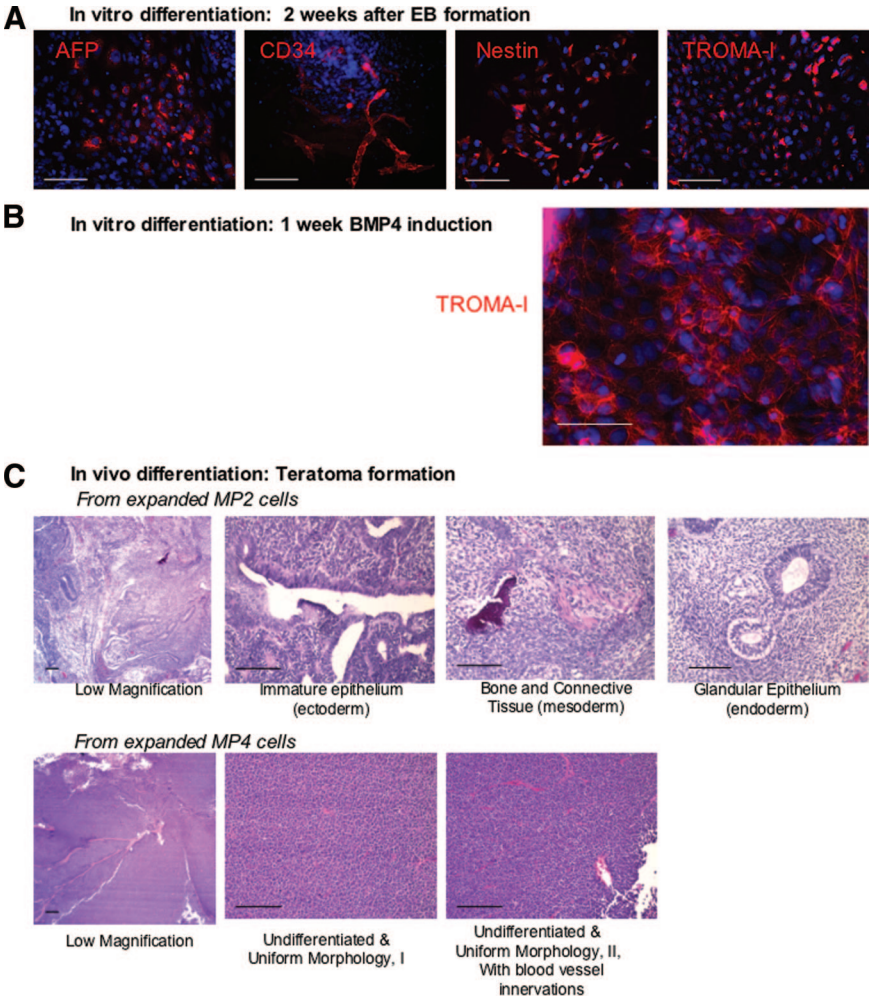

Figure 4. Pluripotency tests of the derived MP2 and MP4 human embryonic stem (hES)-like cells by in vitro and in vivo assays. **(A):** Expanded hES-like cells from MP2 clone (Fig. 2) were induced to differentiate by embryoid body (EB) formation. After 14 days, EBs were broken into small pieces and allowed to grow on a gelatin-coated plate as adherent culture, and then fixed for immunostaining. The expression of representative markers of various lineages, such as AFP (endoderm), CD34 (mesoderm), nestin (ectoderm), and TROMA-I (trophectoderm) were detected. Scale bars = 100 μm in all micrographs. **(B):** MP2 hES-like cells were also induced to differentiate by bone morphogenetic protein 4 for 7 days, under the conditions favoring trophoblast differentiation as used previously for hES cells. Nearly all the cells expressed TROMA-I. **(C):** In vivo differentiation assay by teratoma formation. Cells from either MP2 or MP4 clones were injected in immunodeficient mice. Sections from excised tumors were stained by H&E for histology analysis. Under low magnification (×40), sections from the MP2 samples showed complex and differentiated cells of various types. Under higher magnification (×100), differentiated cells resembling those derived from ectoderm, mesoderm, and endoderm were easily found. In contrast, tumor sections (seven of seven) from the MP4 cells did not show complex structure as MP2 cells did. Under higher magnification, cells appeared more uniform and undifferentiated. Some of them showed more blood vessel innervations (right panel). Abbreviations: AFP, α-fetal protein; BMP, bone morphogenetic protein.

During the course of this study, Park et al. [3] published that adding two genes (TERT and T) to the SY4 cocktail significantly increased iPS efficiency of embryonic or fetal fibroblasts and managed to generate iPS-like colonies at a level of 3–21 per 10^5 postnatal human fibroblasts. This is the first reported attempt to add supplementing factors to enhance the efficiency of reprogramming human postnatal fibroblasts [3]. Although the fibroblasts and T expression vectors that we used were different, our results corroborate those of Park et al. that the generation of human iPS cells can be improved by using additional genes or optimized combinations [3]. We showed that adding T alone to either SY4 or JT4 combination, or even to the common denominator OCT4 and SOX2, would increase efficiency of human iPS cell production by 23–70 fold. However, our results differ in one important aspect. In their report, Park et al. did not detect the integration of retroviral vectors expressing T or hTERT in the hES-like colonies that they ultimately isolated and examined [3]. They speculated that these factors (T and hTERT) might act indirectly on supportive cells in the culture to enhance the efficiency with which the reprogrammed colonies can be selected [3]. In contrast, we detected the presence of the T transgene in each of the 34 hES-like clones we examined, including 12 clones from which T was delivered by NIL vectors. However, the persistence of the T vector did not always inhibit the pluripotency or differentiation potential of isolated hES-like clones such as MP2, but it may contribute an increased level of abnormal karyotypes.

For some of iPS-like cells, such as MP2, that were established by our improved method, we completed pluripotency assays both in vitro and in vivo. We demonstrate for the first time that the human iPS cells, such as MP2, can differentiate in vitro to trophoblasts in addition to the three embryonic germ layers, characteristic of hES cells. Using the in vivo teratoma assay, MP2 iPS cells, like classic hES cells, form complex structures with cells derived from all the three embryonic germ layers. Interestingly, clones that are reprogrammed by the same set of genes and cultured under the same condition, such as MP4, failed to differentiate into any cell types in both in vitro and in vivo assays. In many ways, they resemble nullipotent EC cells that were reported previously [30, 31]. It remains to be determined whether MP4 can be induced to differentiate by chemicals commonly used to induce human EC and ES cells. It is of great interest to elucidate the underlying differences between MP2 and MP4 cell types, which are derived from the same fibroblasts by the same set of reprogramming genes and cultured under the same condition.

SUMMARY

We independently observed that SV40 T antigen can significantly enhance reprogramming of human adult as well as fetal fibroblasts with various transgene combinations. The generated iPS cells resemble classic human ES cells in morphology, marker expression, and differentiation potential to generate cells

derived from the three embryonic germ layers and trophectoderm lineage. The greatly improved efficiency of obtaining human iPS cells will aid our efforts to generate patient-specific pluripotent stem cells and to investigate the underlying mechanisms of reprogramming and its relationship with cellular transformation leading to carcinogenesis.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Improved Efficiency and Pace of Generating Induced Pluripotent Stem Cells from Human Adult and Fetal Fibroblasts

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