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# Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors

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## SUMMARY

Differentiated cells can be reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with embryonic stem (ES) cells. Little is known about factors that induce this reprogramming. Here, we demonstrate induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Unexpectedly, Nanog was dispensable. These cells, which we designated iPS (induced pluripotent stem) cells, exhibit the morphology and growth properties of ES cells and express ES cell marker genes. Subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. These data demonstrate that pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few defined factors.

## INTRODUCTION

Embryonic stem (ES) cells, which are derived from the inner cell mass of mammalian blastocysts, have the ability to grow indefinitely while maintaining pluripotency and the ability to differentiate into cells of all three germ layers (Evans and Kaufman, 1981; Martin, 1981). Human ES cells might be used to treat a host of diseases, such as Parkinson's disease, spinal cord injury, and diabetes (Thomson et al., 1998). However, there are ethical difficulties regarding the use of human embryos, as well as the problem of tissue rejection following transplantation in patients. One way to circumvent these issues is the generation of pluripotent cells directly from the patients' own cells.

Somatic cells can be reprogrammed by transferring their nuclear contents into oocytes (Wilmut et al., 1997) or by fusion with ES cells (Cowan et al., 2005; Tada et al., 2001), indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells. We hypothesized that the factors that play important roles in the maintenance of ES cell identity also play pivotal roles in the induction of pluripotency in somatic cells.

Several transcription factors, including Oct3/4 (Nichols et al., 1998; Niwa et al., 2000), Sox2 (Avilion et al., 2003), and Nanog (Chambers et al., 2003; Mitsui et al., 2003), function in the maintenance of pluripotency in both early embryos and ES cells. Several genes that are frequently upregulated in tumors, such as *Stat3* (Matsuda et al., 1999; Niwa et al., 1998), *E-Ras* (Takahashi et al., 2003), c-*myc* (Cartwright et al., 2005), *Klf4* (Li et al., 2005), and β-*catenin* (Kielman et al., 2002; Sato et al., 2004), have been shown to contribute to the long-term maintenance of the ES cell phenotype and the rapid proliferation of ES cells in culture. In addition, we have identified several other genes that are specifically expressed in ES cells (Maruyama et al., 2005; Mitsui et al., 2003).

In this study, we examined whether these factors could induce pluripotency in somatic cells. By combining four selected factors, we were able to generate pluripotent cells, which we call induced pluripotent stem (iPS) cells, directly from mouse embryonic or adult fibroblast cultures.

## RESULTS

We selected 24 genes as candidates for factors that induce pluripotency in somatic cells, based on our hypothesis that such factors also play pivotal roles in the maintenance of ES cell identity (see Table S1 in the Supplemental Data available with this article online). For β-catenin, c-Myc, and Stat3, we used active forms, S33Y-β-catenin (Sadot et al., 2002), T58A-c-Myc (Chang et al., 2000), and Stat3-C (Bromberg et al., 1999), respectively. Because of the reported negative effect of Grb2 on pluripotency (Burdon et al., 1999; Cheng et al., 1998), we included its dominant-negative mutant Grb2ΔSH2 (Miyamoto et al., 2004) as 1 of the 24 candidates.

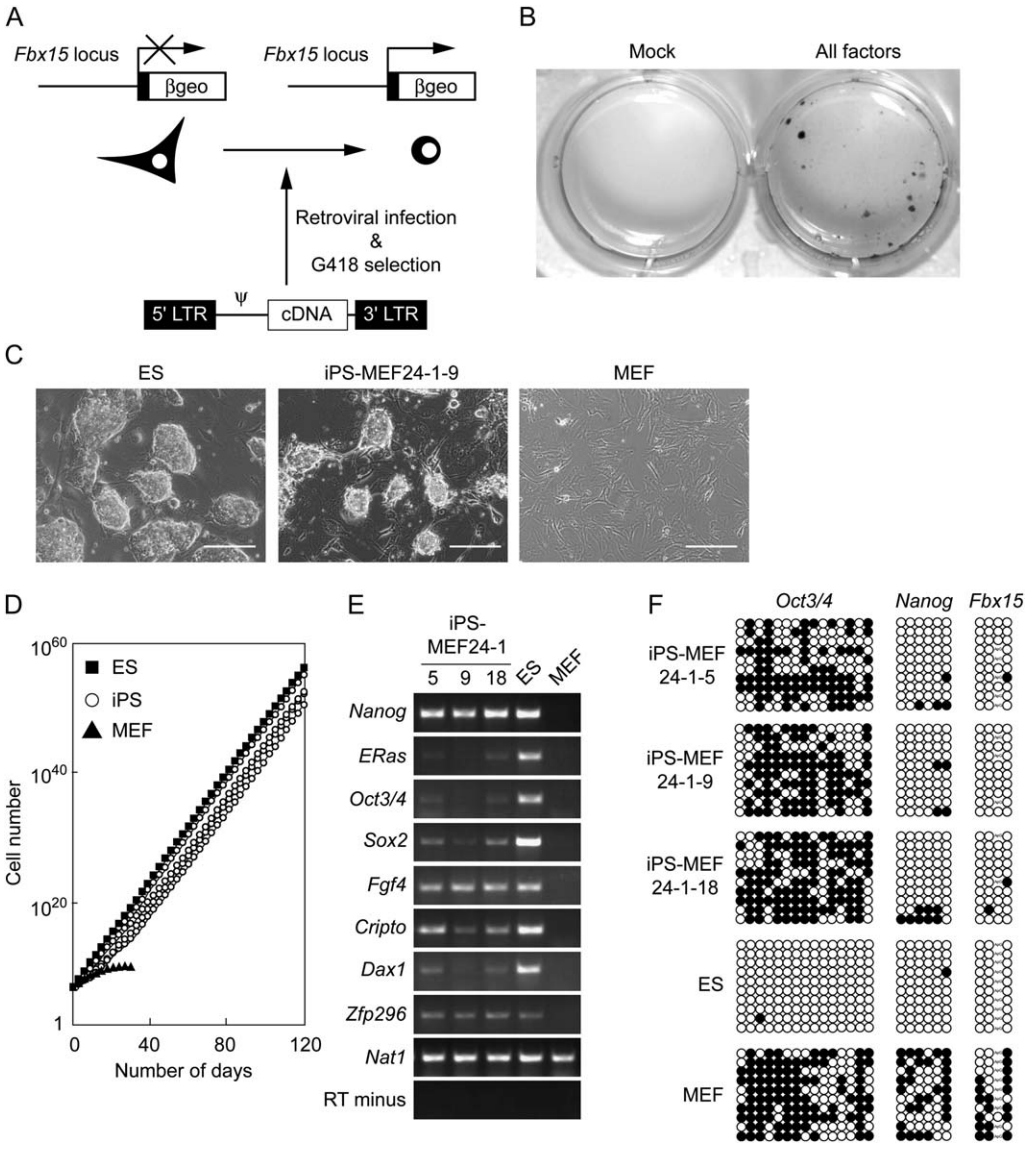


**Figure 1. Generation of iPS Cells from MEF Cultures via 24 Factors**

(A) Strategy to test candidate factors.

(B) G418-resistant colonies were observed 16 days after transduction with a combination of 24 factors. Cells were stained with crystal violet.

(C) Morphology of ES cells, iPS cells (iPS-MEF24, clone 1-9), and MEFs. Scale bars = 200 μm.

(D) Growth curves of ES cells, iPS cells (iPS-MEF24, clones 2-1–4), and MEFs. $3 \times 10^5$ cells were passaged every 3 days into each well of six-well plates.

(E) RT-PCR analysis of ES cell marker genes in iPS cells (iPS-MEF24, clones 1-5, 1-9, and 1-18), ES cells, and MEFs. *Nat1* was used as a loading control.

(F) Bisulfite genomic sequencing of the promoter regions of *Oct3/4*, *Nanog*, and *Fbx15* in iPS cells (iPS-MEF24, clones 1-5, 1-9, and 1-18), ES cells, and MEFs. Open circles indicate unmethylated CpG dinucleotides, while closed circles indicate methylated CpGs.

To evaluate these 24 candidate genes, we developed an assay system in which the induction of the pluripotent state could be detected as resistance to G418 (Figure 1A). We inserted a *βgeo* cassette (a fusion of the β-galactosidase and neomycin resistance genes) into the mouse *Fbx15* gene by homologous recombination (Tokuzawa et al., 2003). Although specifically expressed in mouse ES cells and early embryos, Fbx15 is dispensable for the maintenance of pluripotency and mouse development. ES cells homozygous for the βgeo knockin construct ($Fbx15^{\beta geo/\beta geo}$) were resistant to extremely high concentrations of G418 (up to 12 mg/ml), whereas somatic cells derived from $Fbx15^{\beta geo/\beta geo}$ mice were sensitive to a normal concentration of G418 (0.3 mg/ml). We expected that even partial activation of the *Fbx15* locus would result in resistance to normal concentrations of G418.

We introduced each of the 24 candidate genes into mouse embryonic fibroblasts (MEFs) from $Fbx15^{\beta geo/\beta geo}$

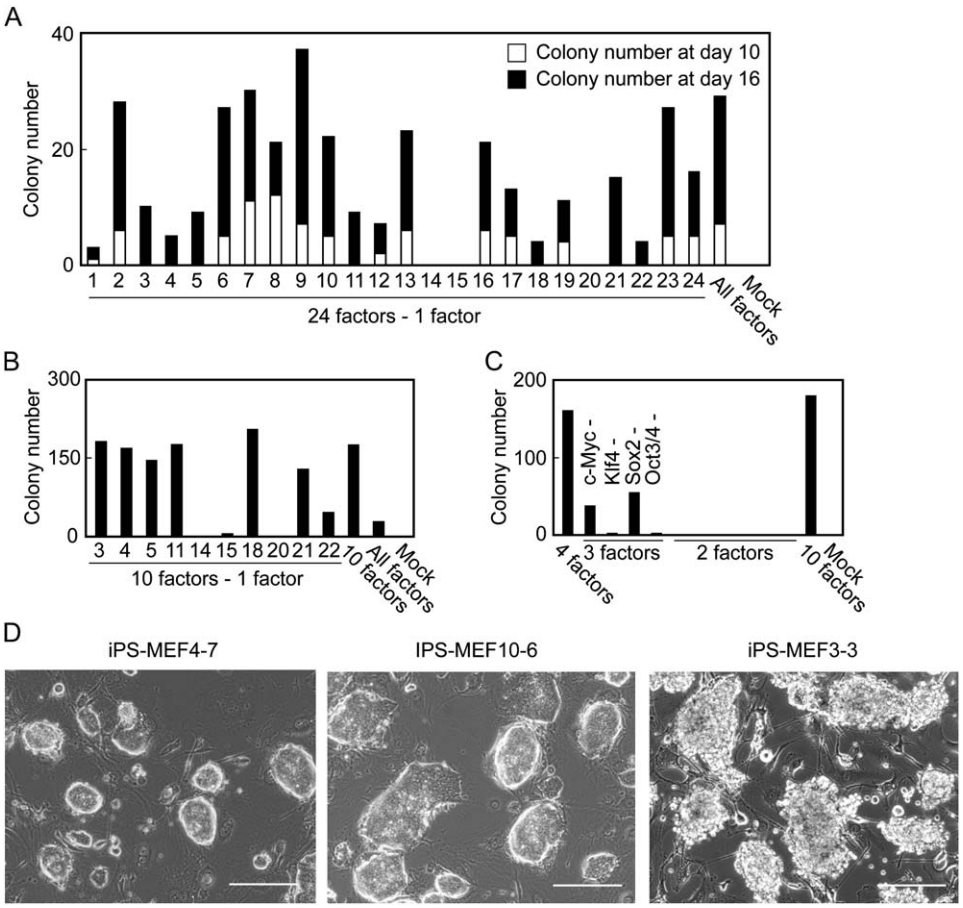


**Figure 2. Narrowing down the Candidate Factors**

(A) Effect of the removal of individual factors from the pool of 24 transduced factors on the formation of G418-resistant colonies. *Fbx15*$^{\beta geo/\beta geo}$ MEFs were transduced with the indicated factors and selected with G418 for 10 days (white columns) or 16 days (black columns).

(B) Effect of the removal of individual factors from the selected 10 factors on the formation of G418-resistant colonies 16 days after transduction.

(C) Effect of the transduction of pools of four, three, and two factors on the formation of G418-resistant colonies 16 days after transduction.

(D) Morphologies of iPS-MEF4 (clone 7), iPS-MEF10 (clone 6), and iPS-MEF3 (clone 3). Scale bars = 200 μm.

embryos by retroviral transduction (Morita et al., 2000). Transduced cells were then cultured on STO feeder cells in ES cell medium containing G418 (0.3 mg/ml). We did not, however, obtain drug-resistant colonies with any single factor, indicating that no single candidate gene was sufficient to activate the *Fbx15* locus (Figure 1B; see also Table S2, which summarizes all of the transduction experiments in this study).

In contrast, transduction of all 24 candidates together generated 22 G418-resistant colonies (Figure 1B). Of the 12 clones for which we continued cultivating under selection, 5 clones exhibited morphology similar to ES cells, including a round shape, large nucleoli, and scant cytoplasm (Figure 1C). We repeated the experiments and observed 29 G418-resistant colonies, from which we picked 6 colonies. Four of these clones possessed ES cell-like morphology and proliferation properties (Figure 1D). The doubling time of these cells (19.4, 17.5, 18.7, and 18.6 hr) was equivalent to that of ES cells (17.0 hr). We designated these cells iPS-MEF24 for "pluripotent stem cells induced from MEFs by 24 factors." Reverse transcription PCR (RT-PCR) analysis revealed that the iPS-MEF24 clones expressed ES cell markers, including *Oct3/4*, *Nanog*, *E-Ras*, *Cripto*, *Dax1*, and *Zfp296* (Mitsui et al., 2003) and *Fgf4* (Yuan et al., 1995) (Figure 1E). Bisulfite genomic sequencing demonstrated that the promoters of *Fbx15* and *Nanog* were demethylated in iPS cells (Figure 1F). By contrast, the *Oct3/4* promoter remained methylated in these cells. These data indicate that some combination of these 24 candidate factors induced the expression of ES cell marker genes in MEF culture.

Next, to determine which of the 24 candidates were critical, we examined the effect of withdrawal of individual factors from the pool of transduced candidate genes on the formation of G418-resistant colonies (Figure 2A). We identified 10 factors (3, 4, 5, 11, 14, 15, 18, 20, 21, and 22) whose individual withdrawal from the bulk transduction pool resulted in no colony formation 10 days after

transduction and fewer colonies 16 days after transduction. Combination of these 10 genes alone produced more ES cell-like colonies than transduction of all 24 genes did (Figure 2B).

We next examined the formation of colonies after withdrawal of individual factors from the 10-factor pool transduced into MEFs (Figure 2B). G418-resistant colonies did not form when either Oct3/4 (factor 14) or Klf4 (factor 20) was removed. Removal of Sox2 (factor 15) resulted in only a few G418-resistant colonies. When we removed c-Myc (factor 22), G418-resistant colonies did emerge, but these had a flatter, non-ES-cell-like morphology. Removal of the remaining factors did not significantly affect colony numbers. These results indicate that Oct3/4, Klf4, Sox2, and c-Myc play important roles in the generation of iPS cells from MEFs.

Combination of the four genes produced a number of G418-resistant colonies similar to that observed with the pool of 10 genes (Figure 2C). We continued cultivation of 12 clones for each transduction and were able to establish 4 iPS-MEF4 and 5 iPS-MEF10 clones. In addition, we could generate iPS cells (iPS-MEF4wt) with wild-type c-Myc instead of the T58A mutant (Table S2). These data demonstrate that iPS cells can be induced from MEF culture by the introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4.

No combination of two factors could induce the formation of G418-resistant colonies (Figure 2C). Two combinations of three factors—Oct3/4, Sox2, and c-Myc (minus Klf4) or Klf4, Sox2, and c-Myc (minus Oct3/4)—generated a single, small colony in each case, but these could not be maintained in culture. With the combination of Oct3/4, Klf4, and Sox2 (minus c-Myc), we observed the formation of 36 G418-resistant colonies, which, however, exhibited a flat, non-ES-cell-like morphology. With the combination of Oct3/4, Klf4, and c-Myc (minus Sox2), we observed the formation of 54 G418-resistant colonies, of which we picked 6. Although all 6 clones could be maintained over several passages, the morphology of these cells (iPS-MEF3) differed from that of iPS-MEF4 and iPS-MEF10 cells, with iPS-MEF3 colonies exhibiting rough surfaces (Figure 2D). These data indicate that the combination of Oct3/4, c-Myc, and Klf4 can activate the *Fbx15* locus, but the change induced by these three factors alone is different from that seen in iPS-MEF4 or iPS-MEF10 cells.

We performed RT-PCR to examine whether ES cell marker genes were expressed in iPS cells (Figure 3A). We used primers that would amplify transcripts of the endogenous gene but not transcripts of the transgene. iPS-MEF10 and iPS-MEF4 clones expressed the majority of marker genes, with the exception of *Ecat1* (Mitsui et al., 2003). The expression of several marker genes, including *Oct3/4*, was higher in iPS-MEF4-7, iPS-MEF10-6, and iPS-MEF10-7 clones than in the remaining clones. *Sox2* was only expressed in iPS-MEF10-6. The iPS-MEF4wt clone also expressed many of the ES cell marker genes (Figure S1). Chromatin immunoprecipitation analyses showed that the promoters of *Oct3/4* and *Nanog* had increased acetylation of histone H3 and decreased dimethylation of lysine 9 of histone H3 (Figure 3B). CpG dinucleotides in these promoters remained partially methylated in iPS cells (Figure 3C). iPS-MEF4 and iPS-MEF10 cells were positive for alkaline phosphatase and SSEA-1 (Figure 3D) and showed high telomerase activity (Figure S2). These results demonstrate that iPS-MEF4 and iPS-MEF10 cells are similar, but not identical, to ES cells.

In iPS-MEF3 clones, *Ecat1*, *Esg1*, and *Sox2* were not activated (Figure 3A). *Nanog* was induced, but to a lesser extent than in iPS-MEF4 and iPS-MEF10 clones. *Oct3/4* was weakly activated in iPS-MEF3-3, -5, and -6 but was not activated in the remaining clones. By contrast, *E-Ras* and *Fgf4* were activated more efficiently in iPS-MEF3 than in iPS-MEF10 or iPS-MEF4. These data confirm that iPS-MEF3 cells are substantially different from iPS-MEF10 and iPS-MEF4 cells.

We compared the global gene-expression profiles of ES cells, iPS cells, and *Fbx15*$^{\beta geo/\beta geo}$ MEFs using DNA microarrays (Figure 4A). In addition, we examined *Fbx15*$^{\beta geo/\beta geo}$ MEFs in which the four factors had been introduced without G418 selection, immortalized MEFs expressing K-RasV12, and NIH 3T3 cells transformed with H-RasV12. Pearson correlation analysis revealed that iPS cells are clustered closely with ES cells but separately from fibroblasts and their derivatives (Figure 4A). The microarray analyses identified genes that were commonly upregulated in ES cells and iPS cells, including *Myb*, *Kit*, *Gdf3*, and *Zic3* (group I, Figure 4B and Table S3). Other genes were upregulated more efficiently in ES cells, iPS-MEF4, and iPS-MEF10 than in iPS-MEF3 clones, including *Dppa3*, *Dppa4*, *Dppa5*, *Nanog*, *Sox2*, *Esrrb*, and *Rex1* (group II). Lower expression of these genes may account for the lack of pluripotency in iPS-MEF3 cells. In addition, we found genes that were upregulated more prominently in ES cells than in iPS cells, including *Dnmt3a*, *Dnmt3b*, *Dnmt3l*, *Utf1*, *Tcl1,* and the *LIF receptor* gene (group III). These data confirm that iPS cells are similar, but not identical, to ES cells.

We examined the pluripotency of iPS cells by teratoma formation (Figure 5A; Table S6 and Figure S3). We obtained tumors with 5 iPS-MEF10 clones, 3 iPS-MEF4 clones, 1 iPS-MEF4wt clone, and 6 iPS-MEF3 clones after subcutaneous injection into nude mice. Histological examination revealed that 2 iPS-MEF10 clones (3 and 6), 2 iPS-MEF4 clones (2 and 7), and the iPS-MEF4wt-4 clone differentiated into all three germ layers, including neural tissues, cartilage, and columnar epithelium. iPS-MEF10-6 could give rise to all three germ layers even after 30 passages (Table S6 and Figure S3). We confirmed differentiation into neural and muscle tissues by immunostaining (Figure 5B) and RT-PCR (Figure S4). By contrast, these teratomas did not express the trophoblast marker *Cdx2* (Figure S4). iPS-MEF10-1 tumors differentiated into ectoderm and endoderm, but not mesoderm, and no signs of differentiation were observed in tumors derived from the remaining iPS-MEF10 (7 and 10) or from iPS-MEF4-10.

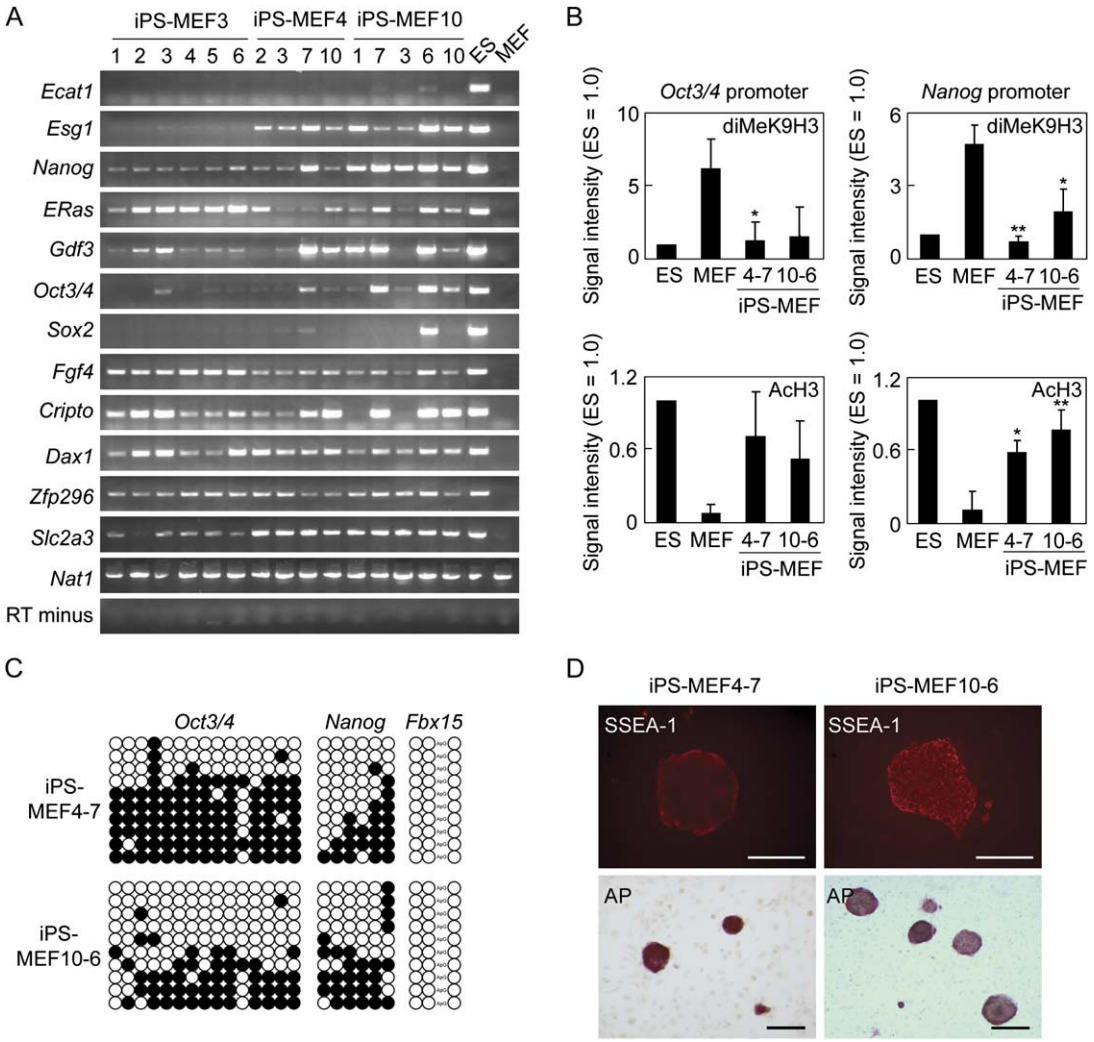


**Figure 3. Gene-Expression Profiles of iPS Cells**

(A) RT-PCR analysis of ES marker genes in iPS cells, ES cells, and MEFs. We used primer sets that amplified endogenous but not transgenic transcripts.

(B) The promoters of *Oct3/4* and *Nanog* were analyzed by ChIP for dimethylation status of lysine 9 of histone H3 and acetylation status of histone H3 in ES cells, MEFs, and iPS cells (MEF4-7 and MEF10-6). Data were quantified by real-time PCR. Shown are the averages and standard deviations of relative values compared to ES cells (n = 3). *$p < 0.05$; **$p < 0.01$ compared to MEFs.

(C) The promoters of *Oct3/4*, *Nanog*, and *Fbx15* were analyzed with bisulfite genomic sequencing for DNA methylation status in iPS-MEF4-7 and iPS-MEF10-6. The DNA methylation status of these promoters in ES cells and MEFs is shown in Figure 1F.

(D) iPS-MEF4-7 and iPS-MEF10-6 clones were stained with a mouse monoclonal antibody against SSEA-1 (480, Santa Cruz) or with an alkaline phosphatase kit (Sigma). Scale bars = 500 μm (SSEA1) and 1 mm (AP).

These data demonstrate that the majority of, but not all, iPS-MEF10 and iPS-MEF4 clones exhibit pluripotency.

In contrast, all tumors derived from iPS-MEF3 clones were composed entirely of undifferentiated cells (Table S6 and Figure S3). Thus, although the three factors (Oct3/4, c-Myc, and Klf4) could induce the expression of some ES cell marker genes, they were not able to induce pluripotency.

iPS-MEF10, iPS-MEF4, and iPS-MEF3 cells formed embryoid bodies in noncoated plastic dishes (Figure 5C). When grown in tissue culture dishes, the embryoid bodies from iPS-MEF10 and iPS-MEF4 cells attached to the dish bottom and initiated differentiation. After 3 days, immunostaining detected cells positive for α-smooth muscle actin (mesoderm marker), α-fetoprotein (endoderm marker), and βIII tubulin (ectoderm marker) (Figure 5D). By contrast, embryoid bodies from iPS-MEF3 cells remained undifferentiated even when cultured in gelatin-coated dishes (Figure 5C). These data confirmed pluripotency of iPS-MEF10 and iPS-MEF4 and nullipotency of iPS-MEF3 in vitro.

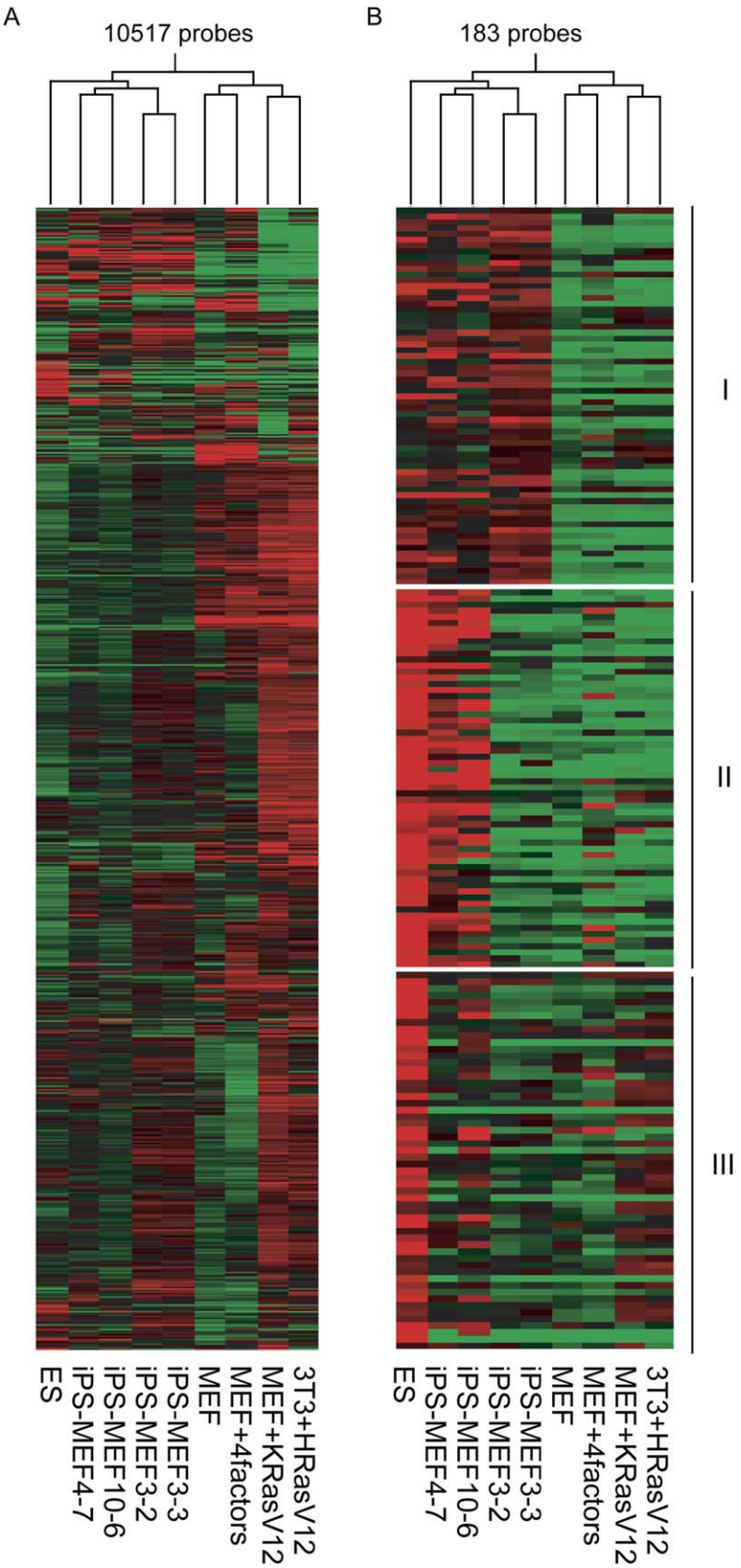


**Figure 4. Global Gene-Expression Analyses by DNA Microarrays**

(A) Pearson correlation analysis of 10,517 probes was performed to cluster ES cells, iPS cells (MEF4- 7, MEF10-6, MEF3-2, and MEF3-3), MEFs, MEFs expressing the four factors, immortalized MEFs expressing K-RasV12, and NIH 3T3 cells transformed by H-RasV12. Red indicates increased expression compared to median levels of the eight samples, whereas green means decreased expression.

(B) Genes upregulated in ES and/or iPS cells. Genes in group I are genes upregulated in ES cells and iPS cells. Genes in group II are upregulated more in ES cells, iPS-MEF4-7, and iPS-MEF10-6 than in iPS-MEF3 cells. Genes in group III are upregulated more in ES cells than in iPS cells. Lists of genes are shown in Tables S3–S5.

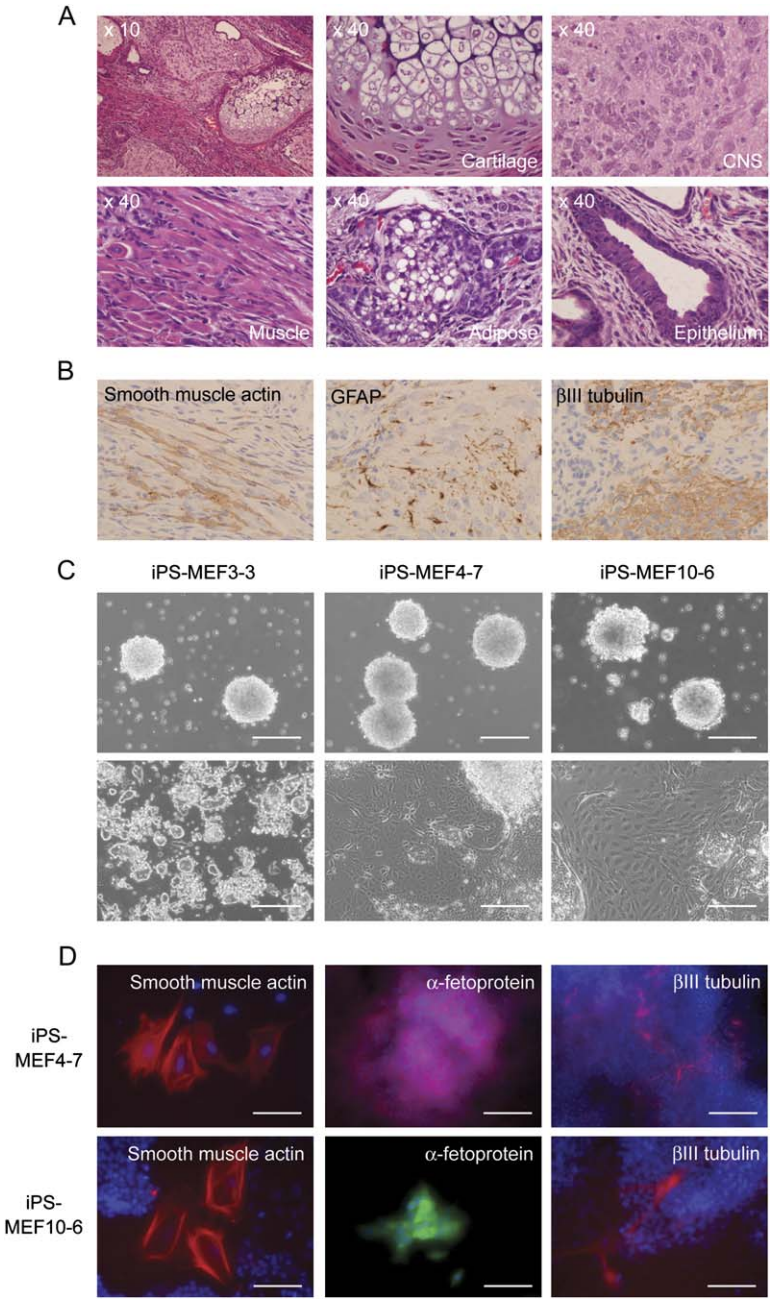


**Figure 5. Pluripotency of iPS Cells Derived from MEFs**

(A) Various tissues present in teratomas derived from iPS-MEF4-7 cells. Histology of other teratomas is shown in Figure S3 and Table S6.

(B) Immunostaining confirming differentiation into neural tissues and muscles in teratomas derived from iPS-MEF4-7.

(C) In vitro embryoid body formation (upper row) and differentiation (lower row). Scale bars = 200 μm.

(D) Immunostaining confirming in vitro differentiation into all three germ layers. Scale bars = 100 μm. Secondary antibodies were labeled with Cy3 (red), except for α-fetoprotein in iPS-MEF10-6, with which Alexa 488 (green) was used.

We next introduced the four selected factors into tail-tip fibroblasts (TTFs) of four 7-week-old male *Fbx15*$^{\beta geo/\beta geo}$ mice on a C57/BL6-129 hybrid background. We obtained 3 G418-resistant colonies, from each of which we could establish iPS cells (iPS-TTF4). We also introduced the four factors into TTFs from a 12-week-old female *Fbx15*$^{\beta geo/\beta geo}$ mouse, which also constitutively expressed green fluorescent protein (GFP) from the CAG promoter and had a C57/BL6-129-ICR hybrid background. Of the 13 G418-resistant colonies obtained, we isolated 6 clones from which we could establish iPS cells (iPS-TTFgfp4, clones 1–6). In addition, we established another iPS-TTFgfp4 (clone 7), in which the cDNA for each of the four factors was flanked with two loxP sites in the transgene. These cells were morphologically indistinguishable from ES cells (Figure 6A). RT-PCR showed that clones 3 and 7 of iPS-TTFgfp4 expressed the majority of ES cell marker genes at high levels and the others at lower levels (Figure 6B). In another attempt, we used either the T58A mutant or the wild-type c-Myc for transduction and established 5 iPS-TTFgfp4 clones (clones 8–12) and 3 iPS-TTFgfp4wt clones (clones 1–3) (Figure S5). RT-PCR

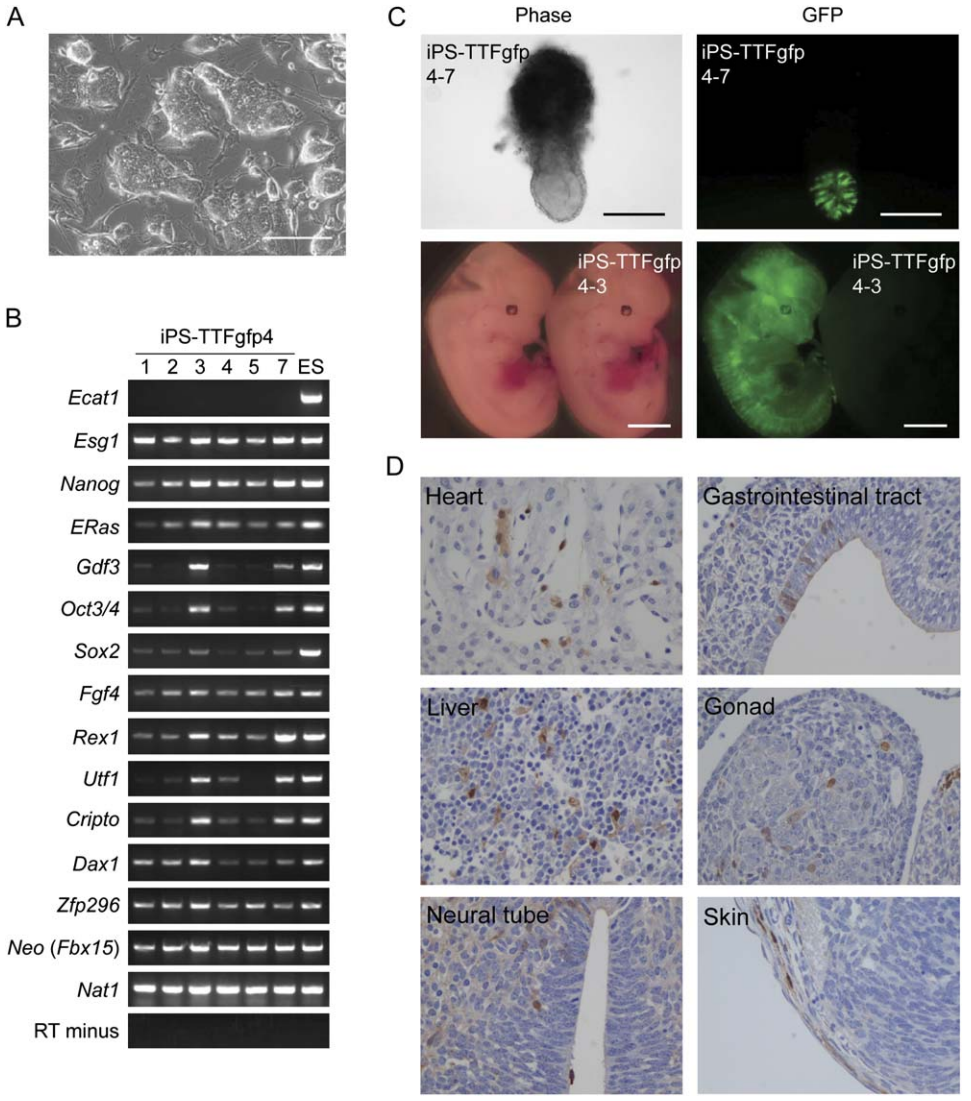


**Figure 6. Characterization of iPS Cells Derived from Adult Mouse Tail-Tip Fibroblasts**

(A) Morphology of iPS-TTFgfp4-3 on STO feeder cells.

(B) RT-PCR analysis of ES marker gene expression in iPS-TTFgfp4 cells (clones 1–5 and 7). We used primer sets that amplified endogenous but not transgenic transcripts.

(C) Contribution of iPS-TTFgfp4-7 and iPS-TTFgfp4-3 cells to mouse embryonic development. iPS cells were microinjected into C57/BL6 blastocysts. Embryos were analyzed with a fluorescence microscope at E7.5 (upper panels, iPS-TTFgfp4-7) or E13.5 (lower panels, iPS-TTFgfp4-3). Scale bars = 200 μm (upper panels) and 2 mm (lower panels).

(D) The E13.5 chimeric embryo was sectioned and stained with anti-GFP antibody (brown). Cells were counterstained with eosin (blue).

showed that iPS-TTFgfp4wt cells also expressed most of the ES cell marker genes (Figure S6).

We transplanted 2 iPS-TTF4 and 6 iPS-TTFgfp4 clones into nude mice, all of which produced tumors containing tissues of all three germ layers (Table S6 and Figure S3). We then introduced 2 clones of iPS-TTFgfp4 cells (clones 3 and 7) into C57/BL6 blastocysts by microinjection. With iPS-TTFgfp4-3, we obtained 18 embryos at E13.5, 2 of which showed contribution of GFP-positive iPS cells (Figure 6C). Histological analyses confirmed that iPS cells contributed to all three germ layers (Figure 6D). We observed GFP-positive cells in the gonad but could not determine whether they were germ cells or somatic cells. With iPS-TTFgfp4-7, we obtained 22 embryos at E7.5, 3 of which were positive for GFP. With the 2 clones, we had 27 pups born, but none of them were chimeric mice. In addition, iPS-TTFgfp4 cells could differentiate into all three germ layers in vitro (Figure S7). These data demonstrate that the four selected factors could induce pluripotent cells from adult mouse fibroblast cultures.

We further characterized the expression of the four factors and others in iPS cells. Real-time PCR confirmed that endogenous expression of *Oct3/4* and *Sox2* was lower in iPS cells than in ES cells (Figure S8). However, the total amount of the four factors from the endogenous genes and the transgenes exceeded the normal expression levels in ES cells. In contrast, Western blot analyses showed that the total protein amounts of the four factors in iPS cells were comparable to those in ES cells (Figure 7A; Figure S8). We could detect Nanog and E-Ras proteins in iPS cells, but at lower levels than those in ES cells (Figures 7A and 7B; Figure S8). The p53 levels in iPS cells were lower than those in MEFs and equivalent to those in ES cells (Figure 7A; Figure S9). The p21 levels in iPS cells varied in each clone and were between those in ES cells and MEFs (Figure S9). Upon differentiation in vitro, the total mRNA expression levels of *Oct3/4* and *Sox2* decreased but remained much higher than in ES cells. In contrast, their protein levels decreased to comparable levels in iPS cells and ES cells (Figure 7B).

Southern blot analyses showed that each iPS clone has a unique transgene integration pattern (Figure 7C). Karyotyping analyses of the iPS-TTFgfp4 (clones 1, 2, 3, 7, and 11) and iPS-TTFgfp4wt (clones 1–3) demonstrated that 2 iPS-TTFgfp4 clones and all of the iPS-TTFgfp4wt clones showed a normal karyotype of 40XX (Figure 7D), while the other 3 iPS-TTFgfp4 clones were 39XO, 40XO +10, and 40Xi(X). Analyses of PCR-based simple sequence length polymorphisms (SSLPs) demonstrated that iPS-MEF clones have a mixed background of C57/BL6 and 129 (Table S7), whereas iPS-TTFgfp clones have a mixed background of ICR, C57/BL6, and 129 (Table S8). Finally, we found that iPS cells could not remain undifferentiated when cultured in the absence of feeder cells, even with the presence of LIF (Figure 7E). These results, together with the different gene-expression patterns, exclude the possibility that iPS cells are merely contamination of preexisting ES cells. Finally, subclones of iPS cells were positive for alkaline phosphatase and could differentiate into all three germ layers in vitro (Figure S10), confirming their clonal nature.

## DISCUSSION

Oct3/4, Sox2, and Nanog have been shown to function as core transcription factors in maintaining pluripotency (Boyer et al., 2005; Loh et al., 2006). Among the three, we found that Oct3/4 and Sox2 are essential for the generation of iPS cells. Surprisingly, Nanog is dispensable. In addition, we identified c-Myc and Klf4 as essential factors. These two tumor-related factors could not be replaced by other oncogenes including E-Ras, Tcl1, β-catenin, and Stat3 (Figures 2A and 2B).

The c-Myc protein has many downstream targets that enhance proliferation and transformation (Adhikary and Eilers, 2005), many of which may have roles in the generation of iPS cells. Of note, c-Myc associates with histone acetyltransferase (HAT) complexes, including TRRAP, which is a core subunit of the TIP60 and GCN5 HAT complexes (McMahon et al., 1998), CREB binding protein (CBP), and p300 (Vervoorts et al., 2003). Within the mammalian genome, there may be up to 25,000 c-Myc binding sites (Cawley et al., 2004), many more than the predicted number of Oct3/4 and Sox2 binding sites (Boyer et al., 2005; Loh et al., 2006). c-Myc protein may induce global histone acetylation (Fernandez et al., 2003), thus allowing Oct3/4 and Sox2 to bind to their specific target loci.

Klf4 has been shown to repress *p53* directly (Rowland et al., 2005), and p53 protein has been shown to suppress *Nanog* during ES cell differentiation (Lin et al., 2004). We found that iPS cells showed levels of p53 protein lower than those in MEFs (Figure 7A). Thus, Klf4 might contribute to activation of *Nanog* and other ES cell-specific genes through *p53* repression. Alternatively, Klf4 might function as an inhibitor of Myc-induced apoptosis through the repression of *p53* in our system (Zindy et al., 1998). On the other hand, Klf4 activates $p21^{CIP1}$, thereby suppressing cell proliferation (Zhang et al., 2000). This antiproliferation function of Klf4 might be inhibited by c-Myc, which suppresses the expression of $p21^{CIP1}$ (Seoane et al., 2002). The balance between c-Myc and Klf4 may be important for the generation of iPS cells.

One question that remains concerns the origin of our iPS cells. With our retroviral expression system, we estimated that only a small portion of cells expressing the four factors became iPS cells (Figure S11). The low frequency suggests that rare tissue stem/progenitor cells that coexisted in the fibroblast cultures might have given rise to the iPS cells. Indeed, multipotent stem cells have been isolated from skin (Dyce et al., 2004; Toma et al., 2001, 2005). These studies showed that ~0.067% of mouse skin cells are stem cells. One explanation for the low frequency of iPS cell derivation is that the four factors transform tissue stem cells. However, we found that the four factors induced iPS cells with comparably low efficiency even from bone marrow stroma, which should be more enriched in mesenchymal stem cells and other multipotent cells (Tables S2 and S6). Furthermore, cells induced by the three factors were nullipotent (Table S6 and Figure S3). DNA microarray analyses suggested that iPS-MEF4 cells and iPS-MEF3 cells have the same origin (Figure 4). These results do not favor multipotent tissue stem cells as the origin of iPS cells.

There are several other possibilities for the low frequency of iPS cell derivation. First, the levels of the four factors required for generation of pluripotent cells may have narrow ranges, and only a small portion of cells expressing all four of the factors at the right levels can acquire ES cell-like properties. Consistent with this idea, a mere 50% increase or decrease in Oct3/4 proteins induces differentiation of ES cells (Niwa et al., 2000). iPS clones overexpressed the four factors when RNA levels were analyzed, but their protein levels were comparable to those in ES cells (Figures 7A and 7B; Figure S8), suggesting that the iPS clones possess a mechanism (or mechanisms) that

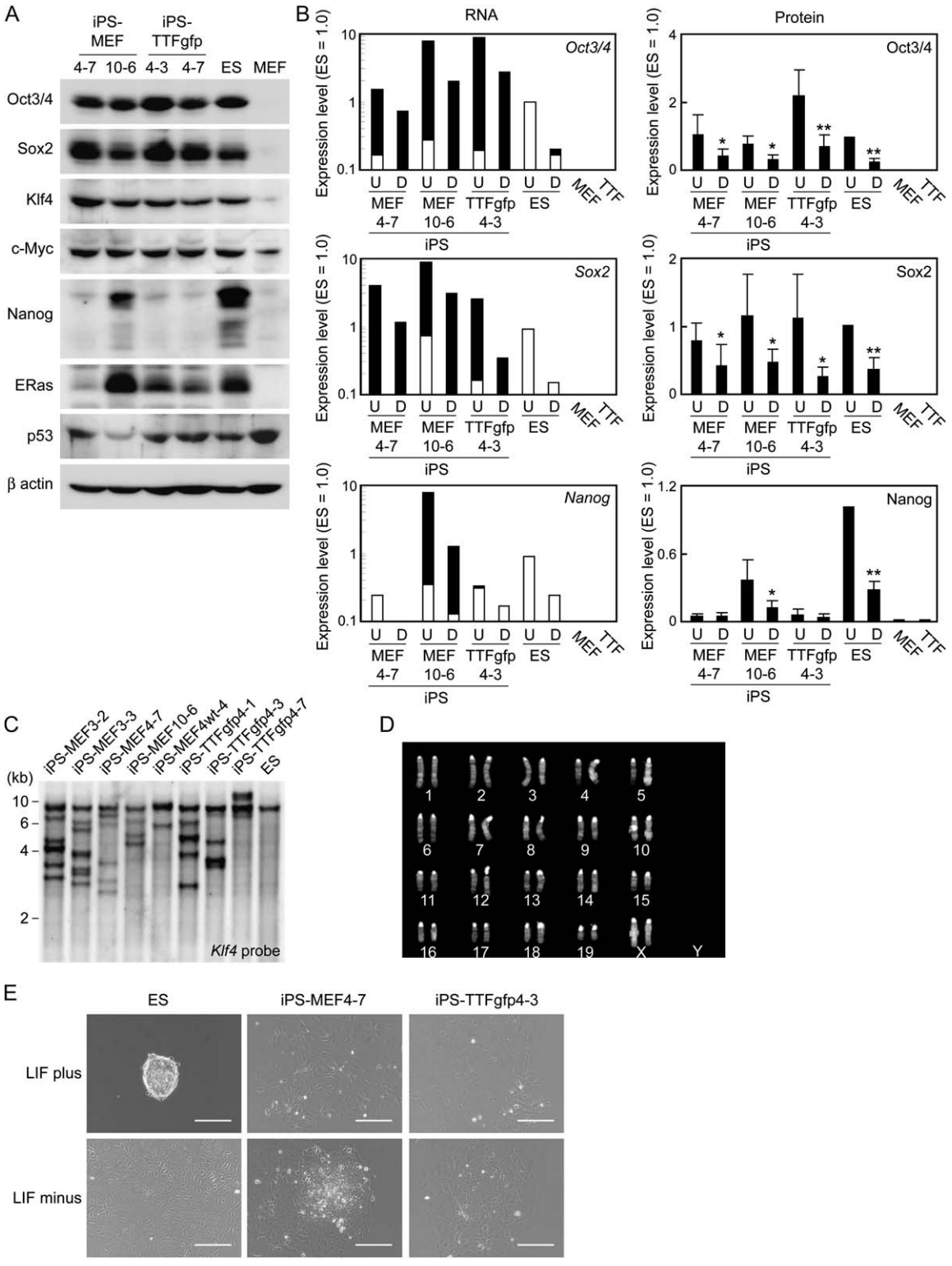


**Figure 7. Biochemical and Genetic Analyses of iPS Cells**

(A) Western blot analyses of the four factors and other proteins in iPS cells (MEF4-7, MEF10-6, TTFgfp4-3, and TTFgfp4-7), ES cells, and MEFs.

(B) Changes in RNA (left) and protein (right) levels of Oct3/4, Sox2, and Nanog in iPS cells and ES cells that were undifferentiated on STO feeder cells (U) or induced to differentiated in vitro through embryoid body formation (D). Shown are relative expression levels compared to undifferentiated ES cells. Data of MEFs and TTFs are also shown. RNA levels were determined with real-time PCR using primers specific for endogenous transcripts

tightly regulates the protein levels of the four factors. We speculate that high amounts of the four factors are required in the initial stage of iPS cell generation, but, once they acquire ES cell-like status, too much of the factors are detrimental for self-renewal. Only a small portion of transduced cells show such appropriate transgene expression. Second, generation of pluripotent cells may require additional chromosomal alterations, which take place spontaneously during culture or are induced by some of the four factors. Although the iPS-TTFgfp4 clones had largely normal karyotypes (Figure 7D), we cannot rule out the existence of minor chromosomal alterations. Site-specific retroviral insertion may also play a role. Southern blot analyses showed that each iPS clone has ~20 retroviral integrations (Figure 7C). Some of these may have caused silencing or fusion with endogenous genes. Further studies will be required to determine the origin of iPS cells.

Another unsolved question is whether the four factors we identified play roles in reprogramming induced by fusion with ES cells or nuclear transfer into oocytes. Since the four factors are expressed in ES cells at high levels, it is reasonable to speculate that they are involved in the reprogramming machinery that exists in ES cells. Our result is also consistent with the finding that the reprogramming activity resides in the nucleus, but not in the cytoplasm, of ES cells (Do and Scholer, 2004). However, iPS cells were not identical to ES cells, as shown by the global gene-expression patterns and DNA methylation status. It is possible that we have missed additional important factors. One such candidate is ECAT1, although its forced expression in iPS cells did not consistently upregulate ES cell marker genes (Figure S12).

More obscure are the roles of the four factors, especially Klf4 and c-Myc, in the reprogramming observed in oocytes. Both Klf4 and c-Myc are dispensable for preimplantation mouse development (Baudino et al., 2002; Katz et al., 2002). Furthermore, c-*myc* is not detected in oocytes (Domashenko et al., 1997). In contrast, L-*myc* is expressed maternally in oocytes. *Klf17* and *Klf7*, but not *Klf4*, are found in expressed sequence-tag libraries derived from unfertilized mouse eggs. Klf4 and c-Myc might be compensated by these related proteins. It is highly likely that other factors are also required to induce complete reprogramming and totipotency in oocytes.

It is likely that the four factors from the transgenes are required for maintaining the iPS cells since the expression of Oct3/4 and Sox2 from the endogenous genes remained low (Figure 7B; Figure S8). We intended to prove this by using transgenes flanked by two loxP sites and obtained an iPS clone (TTF4gfp4-7). However, we noticed that these cells contain multiple loxP sites on multiple chromosomes, and, thus, the Cre-mediated recombination would cause not only deletion of the transgenes but also inter- and intrachromosomal rearrangements. Studies with conditional expression systems, such as the tetracycline-mediated system, are required to answer this question.

We showed that the iPS cells can differentiate in vitro and in vivo even with the presence of the retroviral vectors containing the four factors. We found that Oct3/4 and Sox2 proteins decreased significantly during in vitro differentiation (Figure 7B). Retroviral expression has been shown to be suppressed in ES cells and further silenced upon differentiation by epigenetic modifications, such as DNA methylation (Yao et al., 2004). The same mechanisms are likely to play roles in transgene repression in iPS cells since they express *Dnmt3a*, *3b*, and *3l*, albeit at lower levels than ES cells do (Table S5). In addition, we found that iPS cells possess a mechanism (or mechanisms) that lowers protein levels of the transgenes and Nanog (Figure 7B; Figure S8). The same mechanism may be enhanced during differentiation. However, silencing of *Oct3/4* in iPS-TTFgfp4-3 cells was not complete, which may explain our inability to obtain live chimeric mice after blastocyst microinjection of iPS cells.

An unexpected finding in this study was the efficient activation of *Fgf4* and *Fbx15* by the combination of the three factors devoid of Sox2 since these two genes have been shown to be regulated synergistically by Oct3/4 and Sox2 (Tokuzawa et al., 2003; Yuan et al., 1995). It is also surprising that Nanog is dispensable for induction and maintenance of iPS cells. More detailed analyses of iPS cells will enhance our understanding of transcriptional regulation in pluripotent stem cells.

Our findings may have wider applications, as we have found that transgene reporters with other ES cell marker genes, such as *Nanog*, can replace the *Fbx15* knockin during selection (K. Okita and S.Y., unpublished data). However, we still do not know whether the four factors can generate pluripotent cells from human somatic cells. Use of c-Myc may not be suitable for clinical applications, and the process may require specific culture environments. Nevertheless, the finding is an important step in controlling pluripotency, which may eventually allow the creation of pluripotent cells directly from somatic cells of patients.

## EXPERIMENTAL PROCEDURES

### Mice

*Fbx15*$^{\beta geo/\beta geo}$ mice were generated with 129SvJae-derived RF8 ES cells as described previously (Tokuzawa et al., 2003) and were

(white columns) or those common for both endogenous and transgenic transcripts (white and black columns). RNA expression levels are shown on logarithmic axes. Protein levels were determined by Western blot normalized with β-actin. Protein levels are shown as the averages and standard deviations on linear axes (n = 4). *p < 0.05 compared to undifferentiated cells.

(C) Southern blot analyses showing the integration of transgenes. Genomic DNA isolated from iPS cells and ES cells was digested with EcoRI and BamHI, separated on agarose gel, transferred to a nylon membrane, and hybridized with a *Klf4* cDNA probe.

(D) Normal karyotype of iPS-TTFgfp4-2 clone.

(E) Morphology of ES cells and iPS cells cultured without feeder cells. One thousand cells were cultured on gelatin-coated six-well plates for 5 days, with or without LIF. Scale bars = 200 μm.

backcrossed to the C57/BL6 strain for at least five generations. These mice were used for primary mouse embryonic fibroblast (MEF) and tail-tip fibroblast (TTF) preparations. To generate *Fbx15*$^{\beta geo/\beta geo}$ mice with constitutive expression of GFP, an *Fbx15*$^{\beta geo/\beta geo}$ mouse (C57/BL6-129 background) was mated with an ICR mouse with the *GFP* transgene driven by the constitutive CAG promoter (Niwa et al., 1991). The resulting *Fbx15*$^{\beta geo/+,GFP/+}$ mice were intercrossed to generate *Fbx15*$^{\beta geo/\beta geo,GFP/GFP}$ mice. Nude mice (BALB/Jcl-nu) were purchased from CLEA.

### Cell Culture

RF8 ES cells and iPS cells were maintained on feeder layers of mitomycin C-treated STO cells as previously described (Meiner et al., 1996). As a source of leukemia inhibitory factor (LIF), we used conditioned medium (1:10,000 dilution) from Plat-E cell cultures that had been transduced with a LIF-encoding vector. ES and iPS cells were passaged every 3 days. Plat-E packaging cells (Morita et al., 2000), which were also used to produce retroviruses, were maintained in DMEM containing 10% FBS, 50 units/50 μg/ml penicillin/streptomycin, 1 μg/ml puromycin (Sigma), and 100 μg/ml of blasticidin S (Funakoshi).

For MEF isolation, uteri isolated from 13.5-day-pregnant mice were washed with phosphate-buffered saline (PBS). The head and visceral tissues were removed from isolated embryos. The remaining bodies were washed in fresh PBS, minced using a pair of scissors, transferred into a 0.1 mM trypsin/1 mM EDTA solution (3 ml per embryo), and incubated at 37°C for 20 min. After incubation, an additional 3 ml per embryo of 0.1 mM trypsin/1 mM EDTA solution was added, and the mixture was incubated at 37°C for 20 min. After trypsinization, an equal amount of medium (6 ml per embryo DMEM containing 10% FBS) was added and pipetted up and down a few times to help with tissue dissociation. After incubation of the tissue/medium mixture for 5 min at room temperature, the supernatant was transferred into a new tube. Cells were collected by centrifugation (200 × g for 5 min at 4°C) and resuspended in fresh medium. 1 × $10^6$ cells (passage 1) were cultured on 100 mm dishes at 37°C with 5% $CO_2$. In this study, we used MEFs within three passages to avoid replicative senescence.

To establish TTFs, the tails from adult mice were peeled, minced into 1 cm pieces, placed on culture dishes, and incubated in MF-start medium (Toyobo) for 5 days. Cells that migrated out of the graft pieces were transferred to new plates (passage 2) and maintained in DMEM containing 10% FBS. We used TTFs at passage 3 for iPS cell induction.

### Retroviral Infection

The day before transduction, Plat-E cells (Morita et al., 2000) were seeded at 8 × $10^6$ cells per 100 mm dish. On the next day, pMXs-based retroviral vectors were introduced into Plat-E cells using Fugene 6 transfection reagent (Roche) according to the manufacturer's recommendations. Twenty-seven microliters of Fugene 6 transfection reagent was diluted in 300 μl DMEM and incubated for 5 min at room temperature. Nine micrograms of plasmid DNA was added to the mixture, which was incubated for another 15 min at room temperature. After incubation, the DNA/Fugene 6 mixture was added drop by drop onto Plat-E cells. Cells were then incubated overnight at 37°C with 5% $CO_2$.

Twenty-four hours after transduction, the medium was replaced. MEFs or TTFs were seeded at 8 × $10^5$ cells per 100 mm dish on mitomycin C-treated STO feeders. After 24 hr, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45 μm cellulose acetate filter (Schleicher & Schuell) and supplemented with 4 μg/ml polybrene (Nacalai Tesque). Target cells were incubated in the virus/polybrene-containing supernatants for 4 hr to overnight. After infection, the cells were replated in 10 ml fresh medium. Three days after infection, we added G418 at a final concentration of 0.3 mg/ml. Clones were selected for 2 to 3 weeks.

### Plasmid Construction

To generate pMXs-gw, we introduced a Gateway cassette rfA (Invitrogen) into the EcoRI/XhoI site of the pMXs plasmid. Primers used are listed in Table S9. Mutations in β-*catenin*, c-*myc*, and *Stat3* were introduced by PCR-based site-directed mutagenesis. For forced expression, we amplified the coding regions of candidate genes by RT-PCR, cloned these sequences into pDONR201 or pENTR-D-TOPO (Invitrogen), and recombined the resulting plasmids with pMXs-gw by LR reaction (Invitrogen).

### Teratoma Formation and Histological Analysis

ES cells or iPS cells were suspended at 1 × $10^7$ cells/ml in DMEM containing 10% FBS. Nude mice were anesthetized with diethyl ether. We injected 100 μl of the cell suspension (1 × $10^6$ cells) subcutaneously into the dorsal flank. Four weeks after the injection, tumors were surgically dissected from the mice. Samples were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

### Bisulfite Genomic Sequencing

Bisulfite treatment was performed using the CpGenome modification kit (Chemicon) according to the manufacturer's recommendations. PCR primers are listed in Table S9. Amplified products were cloned into pCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers for each gene.

### Determination of Karyotypes and SSLP by PCR

Karyotypes were determined with quinacrine-Hoechst staining at the International Council for Laboratory Animal Science (ICLAS) Monitoring Center (Japan). We obtained PCR primer sequences for SSLP from the Mouse Genome Informatics website (The Jackson Laboratory, http://www.informatics.jax.org). Allele sizes were approximated on the basis of the known allele sizes in various inbred strains.

### Western Blot Analyses

Western blot was performed as previously described (Takahashi et al., 2003). The primary antibodies used were anti-Oct3/4 monoclonal antibody (C-10, Santa Cruz), anti-Sox2 antiserum (Maruyama et al., 2005), anti-Klf4 polyclonal antibody (H-180, Santa Cruz), anti-c-Myc polyclonal antibody (A-14, Santa Cruz), anti-Nanog antiserum (Mitsui et al., 2003), anti-E-Ras antiserum (Takahashi et al., 2003), anti-p53 polyclonal antibody (FL-393, Santa Cruz), and anti-β-actin monoclonal antibody (A5441, Sigma).

### RT-PCR for Marker Genes

We performed reverse transcription reactions using ReverTra Ace -α- (Toyobo) and the oligo $dT_{20}$ primer. PCR was done with ExTaq (Takara). Real-time PCR was performed with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) according to manufacturer's instructions. Signals were detected with an ABI7300 Real-Time PCR System (Applied Biosystems). Primer sequences are listed in Table S9.

### DNA Microarray

Total RNA from ES cells, iPS cells, or MEFs were labeled with Cy3. Samples were hybridized to a Mouse Oligo Microarray (G4121B, Agilent) according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analyzed using GeneSpring GX software (Agilent).

### In Vitro Differentiation of iPS Cells

Cells were harvested by trypsinization and transferred to bacterial culture dishes in the ES medium without G418 or LIF. After 3 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for another 3 days. The cells were stained with anti-α-smooth muscle actin monoclonal antibody (N1584, Dako), anti-α-fetoprotein polyclonal antibody (N1501, Dako) or anti-βIII tubulin monoclonal antibody (CBL412, Abcam) along with 4′-6-diamidino-2-phenylindole (Sigma). Total RNA derived from plated embryoid bodies on day 6 was used for RT-PCR analysis.

### Chromatin Immunoprecipitation Assay

We performed chromatin immunoprecipitation (ChIP) as previously described (Maruyama et al., 2005). Antibodies used in this experiment were anti-dimethyl K9 H3 rabbit polyclonal antibody (ab7312-100, Abcam) and anti-acetyl H3 rabbit polyclonal antibody (06-599, Upstate). PCR primers are listed in Table S9.

### Statistical Analyses

Data are shown as averages and standard deviations. We used Student's t test for protein-level analyses and one-factor ANOVA with Scheffe's post hoc test for ChIP analyses. All statistical analyses were done with Excel 2003 (Microsoft) with the Statcel2 add-on (OMS).

### Supplemental Data

Supplemental Data include 12 figures and 9 tables and can be found with this article online at http://www.cell.com/cgi/content/full/126/4/663/DC1/.

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## Accession Numbers

Microarray data are available in GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi) with the accession number GSE5259.