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The timing of retroviral silencing correlates with the quality of induced pluripotent stem cell lines

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Abstract

Induced pluripotent stem (iPS) cells that are functionally comparable to embryonic stem (ES) cells can be generated from somatic cells by introducing the four transcription factors Oct4, Sox2, Klf4, and c-Myc using retroviruses. Given that iPS cell technology may be useful for medical applications, the quality of iPS cells needs to be maintained during prolonged cultivation. However, it is unclear whether there are any differences in the quality of stability among different iPS clones. Here, I report the efficient selection of stable iPS cells. The iPS colonies that underwent retroviral silencing on day 14 (called early iPS) were more stable than those silenced on day 30 (called late iPS) in terms of morphology and karyotype. My early iPS cells expressed pluripotency marker genes and showed proliferation efficiency similar to ES cells. Furthermore, they gave rise to adult chimeras and could show germline competency when injected into blastocysts or eight-cell-stage embryos. In contrast, the late iPS cells tended to lose their ES cell-like morphology and normal karyotype in long-term culture. This study is a critical step towards optimizing the iPS technology that can be available for medical applications.

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General Introduction

Derivation of ES cells

Pluripotent stem cells can self-renew and generate all cell types of the body. A major example of pluripotent stem cells is embryonic stem (ES) cells, which are derived from the inner cell mass of blastocysts [1-3]. ES cells express pluripotency marker genes such as Oct4 (also called Oct3/4 or Pou5f1), Sox2, and Nanog, and differentiate into all three (endoderm, mesoderm, and ectoderm) germ layers *in vitro*. Furthermore, mouse and human ES cells form teratomas when injected into immune-deficient mice, and mouse ES cells give rise to adult chimeras and contribute to germline transmission when microinjected into eight-cell-stage embryos or blastocysts. Therefore, ES cells are considered to provide an attractive source in regenerative medicine and developmental biology. When ES cells are utilized for transplantation therapy, however, there are two problems: (i) the derivation of ES cells requires fertilized eggs and developing embryos and (ii) the use of other people's ES cells could result in immunological rejection. To avoid these issues, it would be important to obtain pluripotent stem cells without using others' embryos.

Nuclear transfer

The first nuclear transfer experiments, which involved the transfer of the nuclei into enucleated oocytes, were performed using frog in order to examine whether the nuclei of differentiated cells are equivalent to those of embryonic cells [4, 5]. These studies showed that adult cells could be reprogrammed into pluripotent cells. In 1997, the mammal was first cloned from follicle cells by the generation of the sheep "Dolly" [6]. Furthermore, mice

were produced from lymphoid cells albeit with two-step method that involved the generation of ES cells derived from cloned embryos [7]. Cloned animals from ES cells and neural stem cells were more efficiently generated than that from the terminally differentiated lymphocytes [8]. Therefore, it has been suggested that undefined factors that regulate the reprogramming may exist in the oocytes, and that the differentiation state of the donor cells may affect reprogramming efficiency [9]. More recently, the strategy that did not require the oocytes was reported; pluripotent cells were produced by nuclear transfer using adult cells and zygotes in mouse [10]. Given that it is difficult to obtain unfertilized human oocytes, this strategy might be applicable to human system and major practical concerns would be solved [9]. Nevertheless, however, an ethical problem is that nuclear transfer strategy requires unfertilized or fertilized eggs.

Cell fusion

Mouse somatic nuclei were reprogrammed into the pluripotent state by another strategy, which is cell fusion of somatic cells with embryonic cells. The pluripotency was shown by fusion or electrofusion of thymocytes with embryonic carcinoma cells or ES cells [11, 12]. When the fused cells were transplanted into immune-deficient mice, teratomas consisting tissues from all three germ layers were obtained, indicating that the pluripotency of the cells is dominant. Reprogramming by cell fusion with human ES cells was also demonstrated [13, 14]. Furthermore, it has been reported that nuclear factors may be responsible for reprogramming by cell fusion [15]. There was a possibility that transcription factors important for the pluripotency might be involved in the nuclear factors, given that the fusion of neural stem cells with ES cells that overexpressed Nanog dramatically increased the

number of reprogrammed cell colonies [16]. It has also been reported that self-renewal of mouse ES cells in the absence of leukemia inhibitory factor can be undergone by Nanog overexpression and that Nanog knockout ES cells differentiate spontaneously [17, 18]. However, the mechanisms underlying reprogramming remain elusive and cell fusion strategy requires ES cells.

Generation of iPS cells

Yamanaka and colleagues hypothesized that transcription factors expressed in ES cells might have the ability to reprogram somatic cells into pluripotent stem cells. They tested 24 candidate factors in reprogramming of mouse fibroblasts and demonstrated the technology to "directly" reprogram mouse somatic cells into pluripotent stem cells. In 2006, they found that retroviral introduction of the transcription factors *Oct4*, *Sox2*, *Klf4*, and *c-Myc* into mouse fibroblasts and the selection for Fbx15 expression resulted in the derivation of induced pluripotent stem (iPS) cells that were similar to ES cells regarding morphology, proliferation efficiency, and teratoma formation while DNA methylation profiles of the iPS cells were different from those of ES cells [19]. Furthermore, Fbx15 iPS cells did not express endogenous Oct4, Sox2, and Nanog or expressed them at lower levels than those of ES cells. When transplanted into blastocysts, the iPS cells could contribute chimeric embryos but not adult mice and germline competency. These observations indicated that Fbx15 iPS cells were not fully reprogrammed. Further experiments showed that mouse iPS cells capable of contribution to adult chimeras could be obtained from genetically unmodified fibroblasts [20], suggesting that selection markers are important for the generation of completely reprogrammed iPS cells.

Expressions of the essential pluripotency genes *Oct4* and *Nanog* were used as a selection marker of mouse iPS cells [21, 22]. In contrast to Fbx15 iPS cells, Oct4 iPS and Nanog iPS cells showed the characteristics comparable to those of ES cells in terms of pluripotency genes expression, DNA demethylation patterns of endogenous *Oct4* and *Nanog* promoter regions, and contribution to viable chimera and germline competency. This indicated that Oct4- or Nanog-selected iPS cells were fully reprogrammed ones and that activation of Oct4 or Nanog was more stringent selection marker than that of Fbx15. Different copy numbers of viral transgenes in one iPS clone were detectable, meaning that proper expression levels required for the generation of fully reprogrammed iPS cells is unclear. It is possible that relative expression levels of the individual factors are crucial for complete reprogramming. Moreover, retroviral silencing of the four factors was observed in Oct4 iPS and Nanog iPS cells but not in Fbx15 iPS cells. The data suggest that iPS cells with silenced retroviral factors are fully reprogrammed, which is consistent with a previous study that retroviral expression is silenced in early embryonic cells due to the methyltransferases activation [23]. Therefore, retroviral silencing is one of important criteria in the pluripotent state.

Human fibroblasts were reprogrammed into iPS cells by expression of *Oct4* and *Sox2*, combined with either *Klf4* and *c-Myc* or *Lin28* and *Nanog* [24-27]. Human iPS cells were similar to ES cells regarding gene expression, proliferation efficiency, methylation patterns of promoter regions of *Oct4* and *Nanog*, and teratoma formation. Induction efficiency of human iPS cells was lower than that of mouse iPS cells. Furthermore, reprogramming of human somatic cells was a gradual process compared to that of mouse cells. Recently, more rapid and efficient reprogramming was reported using human keratinocytes

[28], demonstrating that donor cell type is an important factor for the iPS cell production.

These studies indicate that iPS cells could provide a resource for *in vitro* usage, including drug screening and disease modeling [29-31], and cell transplantation therapy. Given the medical applications of iPS cell technology, it will be necessary to efficiently generate stable iPS cells that can maintain the pluripotency during long-term cultivation. Although different iPS clones with silenced retroviral trasgenes are observed in both mouse and human iPS cell induction, it is not clear whether these clones have different quality of stability.

Retroviral silencing in the pluripotent state

Fbx15-selected iPS cells continue to express retroviral genes and Oct4- or Nanog-selected iPS cells do not, indicating that iPS cells with or without silenced retroviral factors are obtained during reprogramming of somatic cells. It is known that iPS cell-like colonies begin to express endogenous Oct4, Sox2, and Nanog on around day 10 after infection of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* retroviruses. This provides a possibility that expression of these four transcription factors may be required only for initial expression of endogenous Oct4, Scx2, and Nanog, and that activation of methyltransferases Dnmt3a and Dnmt3b associated with retroviral silencing [23] may be detectable at early stage of reprogramming. It is also possible that donor somatic cells may undergo retroviral silencing after the cells are fully reprogrammed, considering that retroviruses are strong targets for silencing in early embryonic cells.

Oct4 iPS and Nanog iPS cells can maintain their pluripotency without expression of the exogenous factors, and excess expression of the retroviral genes could cause

differentiation of pluripotent cells, meaning that observation of retroviral silencing is important for the generation of iPS cells. Total amounts of exogenous and endogenous genes are a critical factor for maintenance of the pluripotency, and thus iPS cells with silenced viral genes may show the characteristics similar to ES cells. Furthermore, endogenous pluripotency genes can be expressed by transcriptional activation of retroviral genes but these viral genes may not be silenced by activation of endogenous Oct4, Scx2, and Nanog, given that retroviral silencing occurs due to epigenetic modification. In addition, retrovirus elements including the region of long terminal repeat may result in increase the risk of tumor formation. Therefore, retroviral silencing can be one of important criteria for the establishment of fully reprogrammed iPS cells that can maintain the pluripotency (Fig. 0).

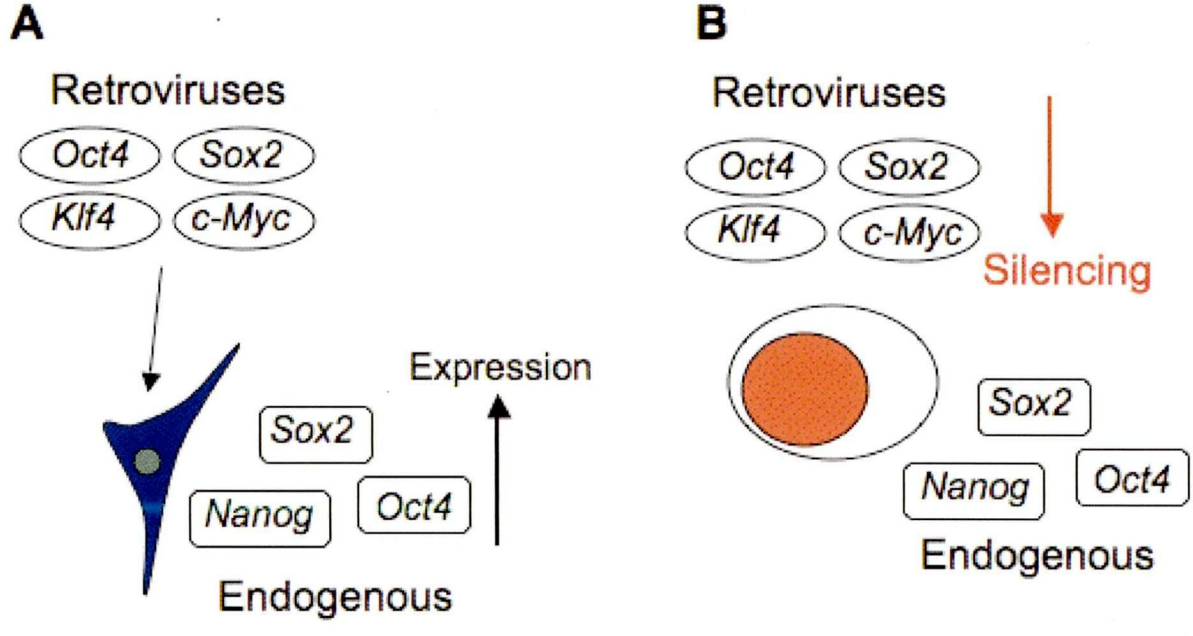


Figure 0. The significance of retroviral silencing in iPS cell generation.

(A) In donor somatic cell, endogenous pluripotency genes such as *Oct4*, *Sox2*, and *Nanog* are expressed by transcription activation of *Oct4*, *Sox2*, *Klf4*, and *c-Myc* retroviruses.

(B) When donor cell is reprogrammed into ES-like cell, total expression of pluripotency genes needs to be conserved in order to maintain the pluripotency.

Introduction

Toward medical applications of iPS cells

Mouse iPS cells were produced from fibroblasts using *Oct4*, *Sox2*, *Klf4*, and *c-Myc* retroviruses [19]. Although *Oct4* and *Sox2* genes are known to be required for maintenance of pluripotency, the roles of *Klf4* and *c-Myc* in somatic cell reprogramming are not fully understood. Given that *L-Myc* could be used instead of *c-Myc* in iPS cell production [32], transformation of donor cells may not be essential for reprogramming. c-Myc was dispensable for mouse cell reprogramming but was crucial for rapid and efficient generation of germline transmittable iPS cells [33]. The proto-oncogene *c-Myc* caused tumor formation in iPS cell-derived chimeric mice and their offspring [22]. Thus, the use of *c-Myc* as a reprogramming factor enhances both iPS cell derivation and tumor formation. Retrovirus results in higher reprogramming efficiency than that obtained using other factor delivery methods reported to date while other reprogramming methods such as adenoviruses [34, 35], plasmid vectors [36], and recombinant proteins [37, 38] have been studied. In the generation of iPS cells with retroviruses, fully reprogrammed iPS cells silenced retroviral expression, whereas incompletely reprogrammed cells continued to express the viral transgenes [22]. Therefore, retroviral silencing is a key feature for the pluripotent state [9, 39]. Moreover, it is reported that the expression of the four exogenous factors Oct4, Sox2, Klf4, and c-Myc is required for at least 10 to 12 days during the reprogramming of mouse fibroblasts [40, 41], and some iPS clones begin to undergo retroviral silencing on around day 10 after infection of the four factors [42]. It is likely that there may be a correlation between the timing of endogenous pluripotency marker expression and viral silencing. At least in mouse iPS cell

induction, retroviral silencing occurred at different time points in individual iPS clones [42]. However, little is known about differences in the quality of stability among different iPS clones undergoing retroviral silencing. If iPS colonies, which silenced viral expression or expressed endogenous Oct4 or Nanog, lose the pluripotency during several passages, the cells are not superior and useful for medical applications *in vitro* and *in vivo*. Thus, it is important to determine efficient methods for stable iPS cell generation.

Retroviral silencing in iPS cell generation

Expression levels of individual pluripotency genes and relative amounts of these genes may be important for maintenance and induction of the pluripotency. For example, in ES cells, upregulation of Oct4 resulted in spontaneous differentiation into primitive endoderm and mesoderm, and down regulation of Oct4 caused differentiation into trophectoderm [43]. In iPS cell induction using retroviruses, although endogenous Oct4 is one of target genes of retroviral transcription factors, it does not regulate the expression of retroviral transgenes. If viral genes continue to express after endogenous Oct4 activation required for maintenance of pluripotency, iPS cells could differentiate into endoderm and mesoderm due to excess expression of Oct4. Alternatively, if viral silencing occurs before sufficient activation of endogenous Oct4, fully reprogrammed iPS cells could not be generated. Therefore, timing of retroviral silencing would be important for iPS cell derivation. Furthermore, incompletion of retroviral silencing could result in increase of tumor formation risk and might prevent epigenetic regulation such as DNA demethylation in treated cells. In this study, the quality of stability of different iPS clones that silenced the retroviral genes was examined.

Materials and Methods

Cell culture

Mouse embryonic fibroblasts (MEFs) and adult tail tip fibroblasts (TTFs) were isolated from E13.5 C57BL/6 embryos and adult C57BL/6 mice (6-7 weeks of age), respectively. MEFs and TTFs were cultured in medium containing Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich) with 10% fetal bovine serum (FBS, Invitrogen), 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. C57BL/6 ES cells were obtained from American Type Culture Collection (SCRC-1002, ATCC). ES and iPS cells were maintained in ES medium [Knockout DMEM (Invitrogen) with 20% Knockout Serum Replacement (Invitrogen), 2 mM L-glutamine, 100 μM nonessential amino acids, 100 μM beta-mercaptoethanol, 1000 units ml^{-1} leukemia inhibitory factor (LIF), 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin] on feeder cells as previously described [42]. These culture media were replaced every day.

Feeder cells

MEFs at passage 2-4 were cultured on 0.15% gelatin until the cells became 90% confluent. Then, 12 μg ml^{-1} mitomycin-C (Kyowa-Kirin, Japan) was added and the cultures were incubated for 2.5 h at 37°C and 5% CO_2. The cells were washed two times with phosphate-buffered saline (PBS) and cultured in medium containing DMEM with 10% FBS, 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin.

Reprogramming

Plat-E packaging cells and the pMX retroviral vectors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*)

were obtained from Addgene. Plat-E cells were cultured in medium containing DMEM with 10% FBS, 50 units ml^{-1} penicillin, and 50 $\mu g\ ml^{-1}$ streptomycin with 1 $\mu g\ ml^{-1}$ puromycin (Sigma-Aldrich) and 1 $\mu g\ ml^{-1}$ blastcidine S (Funakoshi, Japan). Retroviral infection for iPS cell generation was performed as previously described by other reports [19, 44, 45] with minor modifications. MEFs and adult TTFs were infected with viral supernatants generated from transfection (FuGENE 6, Roche) of Plat-E cells with the pMXs. On the next day, the infected MEFs and TTFs (3,000 cells) were re-seeded into ten 10-cm dishes with feeders. The cells were subsequently cultured in ES medium.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIZOL (Invitrogen), and first-strand cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR was performed with KOD plus (Toyobo, Japan) and the products were resolved by electrophoresis with TAE based gel containing 2% agarose.

Immunofluorescence

iPS cells were fixed in 4% paraformaldehyde for 15 min at 25°C, washed three times with PBS, and blocked for 20 min in PBS containing 5% FBS and 0.1% Triton X-100 (Sigma-Aldrich). Primary antibodies against Oct4 (SC-5279, Santa Cruz) or Nanog (RCAB0001P, ReproCELL, Japan) in PBS containing 1% FBS and 0.1% Triton X-100 were applied for 1 h at 25°C. After washing three times with PBS, secondary antibodies were applied for 1 h at 25°C.

Alkaline phosphatase staining

Each iPS clone (1×10^5 cells) was seeded into a well of a six-well plate with feeder cells. Two days later, cells were stained using an alkaline phosphatase kit (Sigma-Aldrich), according to the manufacturer's instructions.

Karyotyping

Karyotyping was performed as described by Longo *et al.* [46] with some modifications. Slides were washed with detergent and rinsed in 70% ethanol. iPS cells (4×10^5 cells in 2 ml of ES medium) were seeded into a well of a gelatin-coated six-well plate. On the next day, 20 μl of colcemid solution (15210-040, Invitrogen) was added and the culture was incubated for 1.5 h at 37°C and 5% CO_2. The cells were washed with PBS, harvested by trypsinization, and centrifuged. The pellet was washed with PBS and resuspended gently in 1 ml of 1% (wt/vol) tri-sodium citrate solution (SCS). One milliliter of SCS was added, and the tube was inverted twice. An additional 6 ml of SCS was added, the tube was inverted twice, and was left for 5 min at room temperature (RT). After centrifugation, 7 ml of supernatant was discarded and the pellet was resuspended gently two times in the remaining 1 ml of SCS. Five drops of fixative (1:3 acetic acid:methanol) were added and the cells were resuspended. Seven milliliters of fixative was added, the tube was vigorously inverted 10 times, and incubated for 15 min at RT (with one inversion during the incubation). The cells were centrifuged, resuspended in 7 ml of fixative, and centrifuged again. Six milliliters of supernatant was removed and the cells were resuspended in the remaining 1 ml of fixative.

The slides were put over a water bath at 37°C. The cells (5–10 drops) were put

onto the slides and exposed for 1 min to steam. The air-dried samples were stained with Hoechst 33342 (Invitrogen) and observed using fluorescent microscopy (400×). Seven chromosomal spreads were counted and if the number of spreads with a normal karyotype of 40 chromosomes was less than four (or 70%), an additional four spreads were counted. The ratio of cells with a normal karyotype was calculated from seven or eleven spreads for each iPS clone.

Generation of chimeras

Two-cell-stage mouse embryos derived from ICR mice were flushed and cultured in Hepes-buffered potassium simplex optimized medium (KSOM) until the eight-cell-stage or early blastocyst stage. iPS cells (6-10 cells) were microinjected into eight-cell-stage embryos or blastocysts. The eight-cell-stage embryos were cultured until the blastocyst stage. These blastocysts were transplanted into the uterine horns of pseudo-pregnant ICR recipients. Chimerism was estimated by coat color contribution. Male Chimeras derived from C57BL/6 (black coat color) iPS cells were mated with ICR (white color) females and germline transmission of iPS cells was indicated by obtaining offspring with black or agouti coat color.

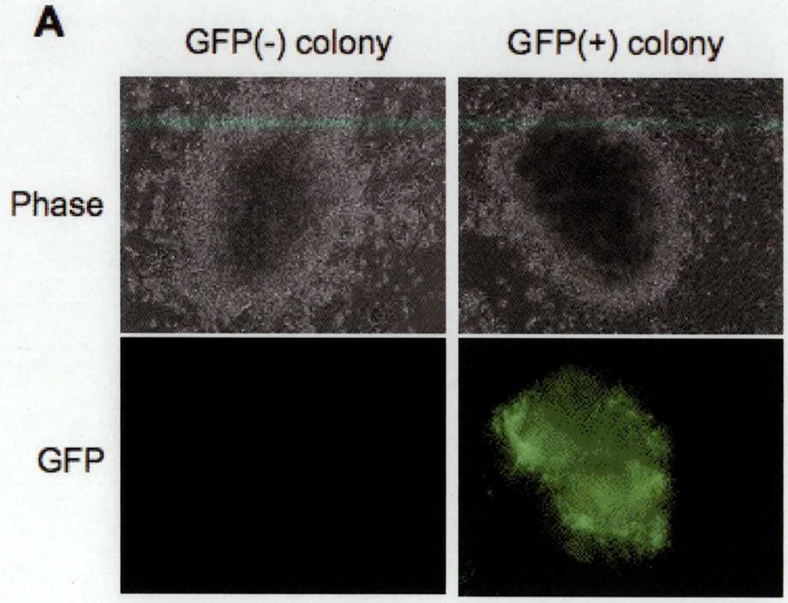
Statistical analysis

The student's *t* test (two-tailed) was used for all statistical analyses.

Results

Induction of iPS colonies

I first assessed the effects of retroviral silencing on gene expression in induced colonies. I infected MEFs using retroviruses expressing *Oct4*, *Sox2*, *Klf4*, *c-Myc*, and green fluorescent protein (*GFP*). I obtained morphologically ES cell-like and GFP-negative or GFP-positive colonies (passage 0). I randomly picked two GFP-negative and four GFP-positive colonies on day 13 after viral infection (Fig. 1A), and performed reverse transcription-polymerase chain reaction (RT-PCR) analysis at passage 3 (Fig. 1B). GFP^- colonies expressed pluripotency marker genes at levels similar to those in ES cells. In contrast, GFP^+ colonies failed to express these markers or expressed them at extremely low levels. Furthermore, GFP^- colonies lost the expression of all of the transgenes whereas GFP^+ colonies still expressed them, indicating that the lack of exogenous GFP expression reflects retroviral silencing. These data suggest that superior iPS clones can be efficiently obtained from GFP^- colonies.



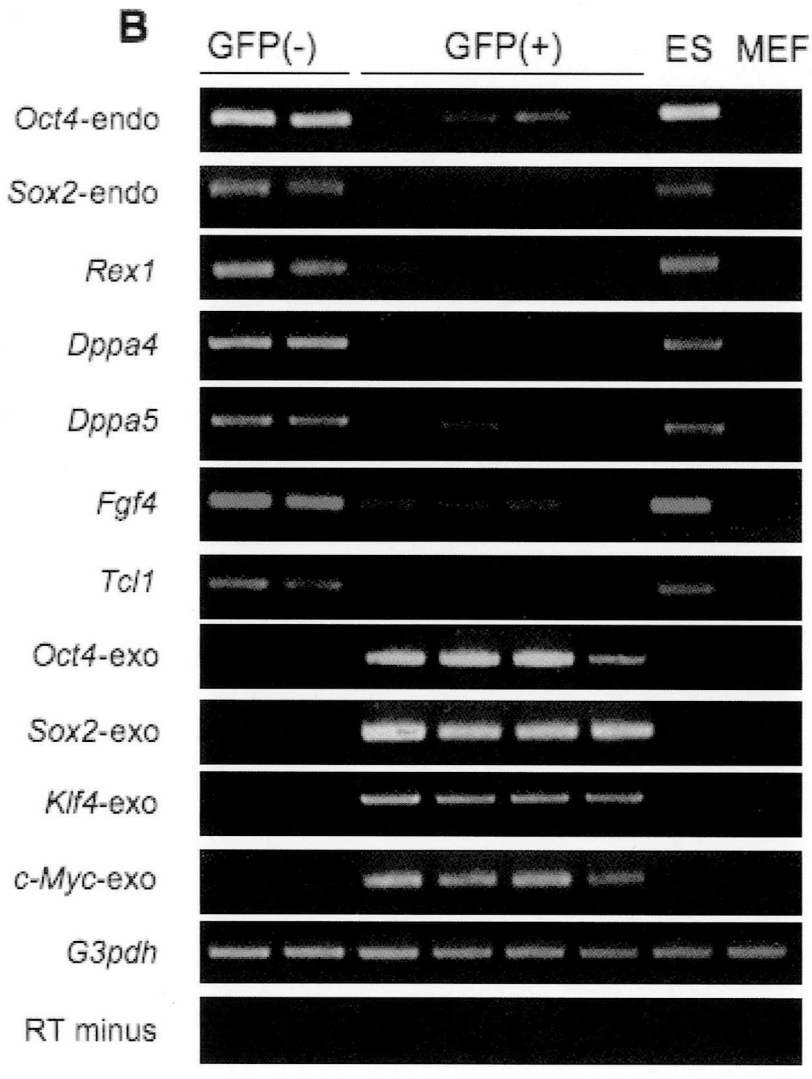


Figure 1. Morphology and gene expression of induced colonies.

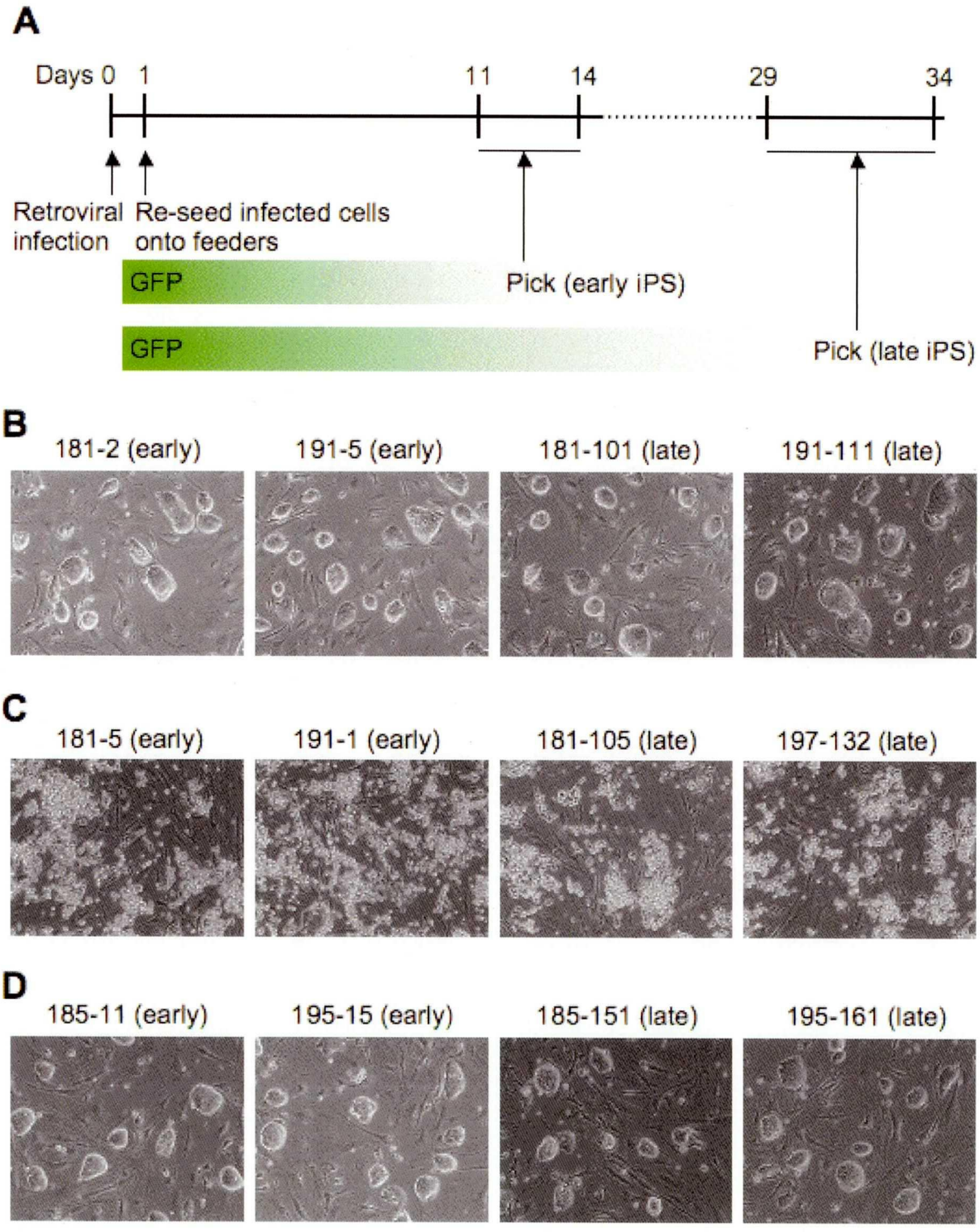
(A) Morphology and GFP fluorescence images. GFP-negative and GFP-positive colonies are shown. (B) RT-PCR analysis. The expression of ES cell markers and transgenes in six clones at passage 3 (two GFP-negative and four GFP-positive clones) was examined. ES cells were used as a control.

Stability in morphology of iPS clones

We previously reported that individual ES cell-like colonies underwent retroviral silencing at different time points, when published culture conditions were used for

reprogramming of mouse fibroblasts [42]. In the present study, I hypothesized that different ES cell-like colonies with silenced retroviral GFP might show different quality; I therefore asked whether the timing of retroviral silencing marks the stability of iPS cells. I infected MEF and adult TTF cells derived from C57BL/6 mice with retroviruses encoding the four factors and *GFP* (Fig. 2A). On the next day, I re-seeded the MEFs and TTFs onto feeder cells. I marked ES cell-like and GFP-positive colonies on day 6, and followed GFP expression every day using fluorescent microscopy. I then attempted to divide the induced colonies into two groups: ES cell-like colonies that underwent retroviral silencing (i) on around day 14 (called early iPS) or (ii) on around day 30 (called late iPS), after infection. Indeed, I picked colonies on days 11-14 or 29-34, when GFP-negative colonies were induced from GFP-positive ones (Fig. 2A and Table 1).

I expanded these clones and continued monitoring their morphology for up to 20 passages (Fig. 2B-E and Table 1). I found a significant difference in the stability of early and late iPS clones. That is, I observed that 21 of 25 early MEF iPS clones maintained ES cell-like morphology at passage 20 (Fig. 2F). In contrast, only 10 of 25 late MEF iPS clones maintained ES cell-like morphology (Fig. 2F). I also observed that 17 of 18 early TTF iPS clones maintained their morphology during 20 passages, whereas only nine of 22 late TTF iPS clones maintained their morphology (Fig. 2G). Furthermore, RT-PCR showed that the expression levels of *Oct4* and *Nanog*, pluripotency markers, in the non-ES-like clones were lower than those in ES cells (Fig. 2H). These data indicate that early iPS clones are more stable than late iPS clones.


A
Days 0
1
11
14
29
34
Retroviral infection
Re-seed infected cells onto feeders
GFP
Pick (early iPS)
GFP
Pick (late iPS)
B
181-2 (early)
191-5 (early)
181-101 (late)
191-111 (late)
C
181-5 (early)
191-1 (early)
181-105 (late)
197-132 (late)
D
185-11 (early)
195-15 (early)
185-151 (late)
195-161 (late)

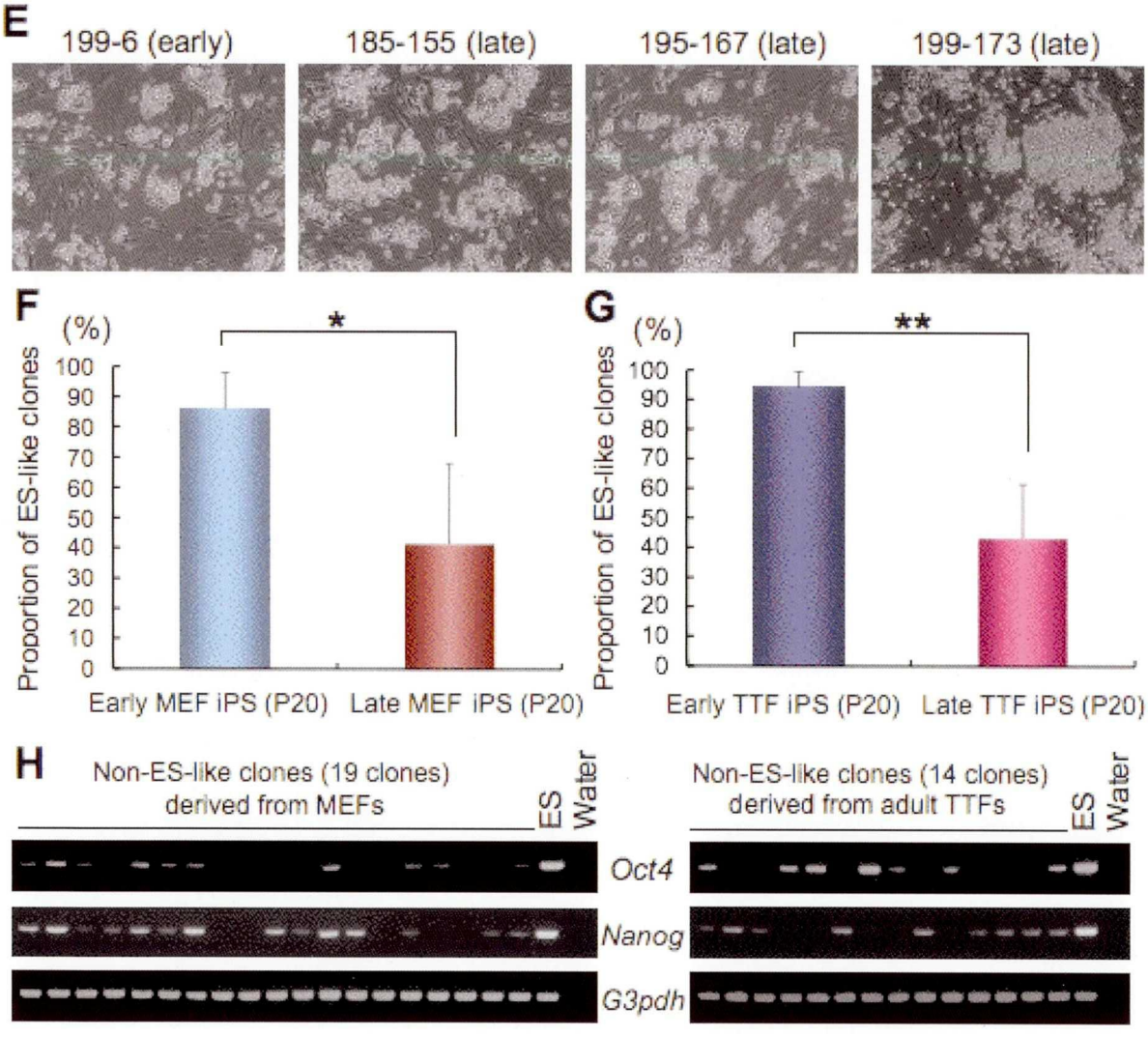


Figure 2. Stability of iPS cell morphology. (A) Outline of early and late iPS production. Early iPS clones (GFP-negative colonies) were picked on days 13, 12, 14, 12, 11, and 14 in experiments number 181, 191, 197, 185, 195, and 199, respectively. Late iPS clones (GFP-negative colonies) were picked on days 30, 34, 31, 31, 34, and 29 in experiments number 181, 191, 197, 185, 195, and 199, respectively. (B-E) Morphology of clones expanded. (B) ES-like early MEF iPS (clones 181-2 and 191-5) and late MEF iPS (clones 181-101 and 191-111). (C) Non-ES-like early MEF iPS (181-5 and 191-1) and late MEF iPS (clones 181-105 and 197-132). (D) ES-like early TTF iPS (clones 185-11 and 195-15) and late TTF iPS (clones 185-151 and 195-161). (E) Non-ES-like early TTF iPS (clone 199-6) and late TTF iPS (clones 185-155, 195-167, and 199-173). (F, G) Stability in morphology of MEF and TTF iPS clones. The ratio at passage 20 was obtained by dividing the number of ES-like clones by the total number of clones expanded (error bars indicate standard deviations; $*p = 0.058$; $**p < 0.01$). (H) RT-PCR. *Oct4* and *Nanog* expression of non-ES-like clones derived from MEFs and from adult TTFs was examined.

Table 1. Establishment of iPS clones

Experiment number	Origin	Days of retroviral silencing	Picked up colonies	ES-like clones at passage 10	ES-like clones at passage 20
181	MEF	13	9	8	7
		30	8	4	4
191		12	10	8	8
		34	8	6	5
197		14	6	6	6
		31	9	6	1
185	TTF	12	6	6	6
		31	6	5	3
195		11	6	6	6
		34	9	7	3
199		14	6	6	5
		29	7	6	3

Similarity of iPS clones with ES cells

I next analyzed the proliferation rates and gene expression levels in the early and late iPS clones that maintained ES cell-like morphology. From four independent experiments (181, 191, 185, and 195), I randomly selected 24 clones: six early MEF iPS (clones 181-1, -2, and -3; 191-5, -7, and -9), six late MEF iPS (clones 181-101, -102, and -103; 191-111, -113, and -115), six early TTF iPS (clones 185-11, -12, and -13; 195-15, -16, and -17), and six late TTF iPS (clones 185-151, -152, and -153; 195-161, -162, and -163). I seeded 1×10^5 cells of each iPS clone at passage 16 into a well of a six-well plate with feeder cells and expanded the cultures every two days. Most of these clones showed proliferation efficiency similar to that of ES cells (Fig. 3 and Table 2). Only one late TTF iPS clone (195-163) showed a longer doubling time (Table 2), suggesting that this clone is not a superior iPS line.

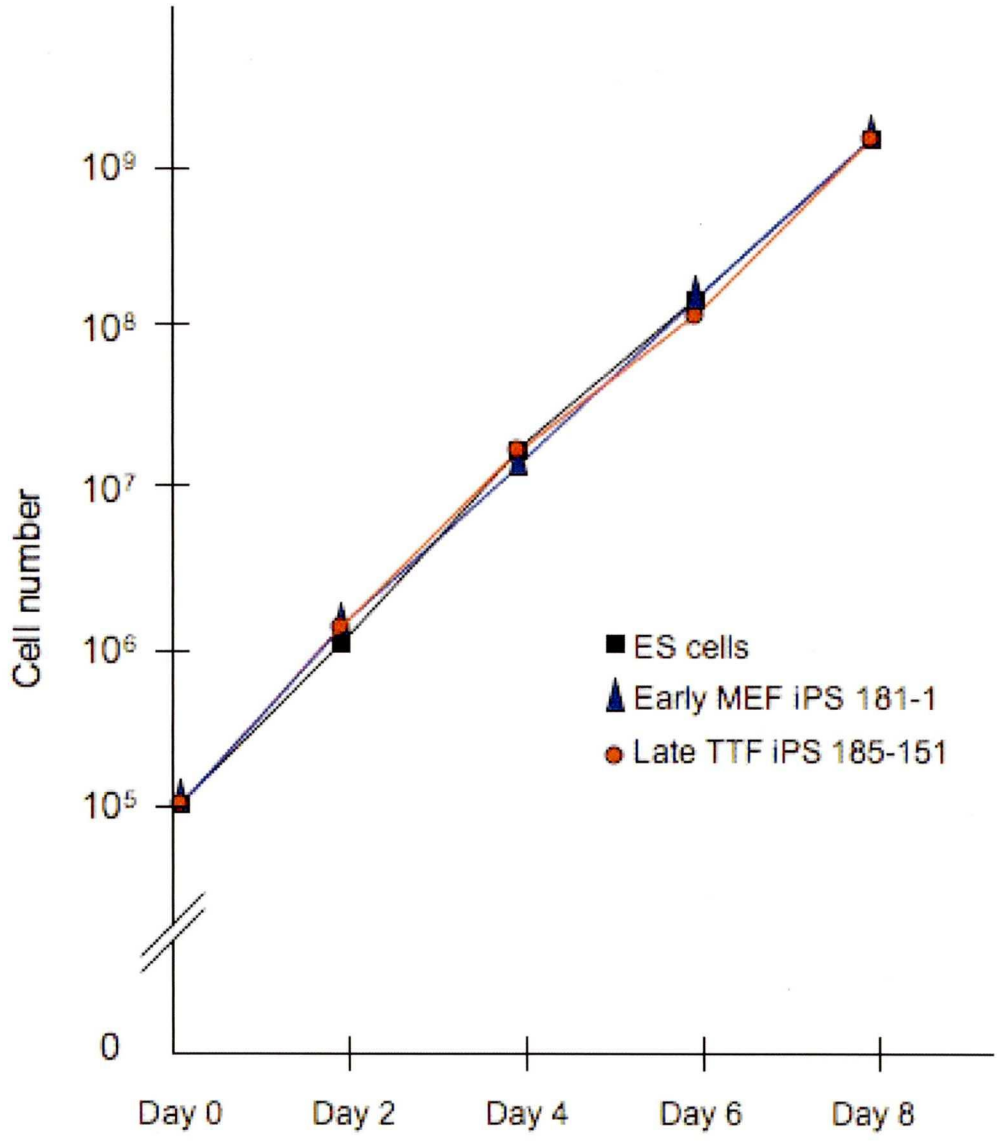


Figure 3. Proliferation of iPS clones.

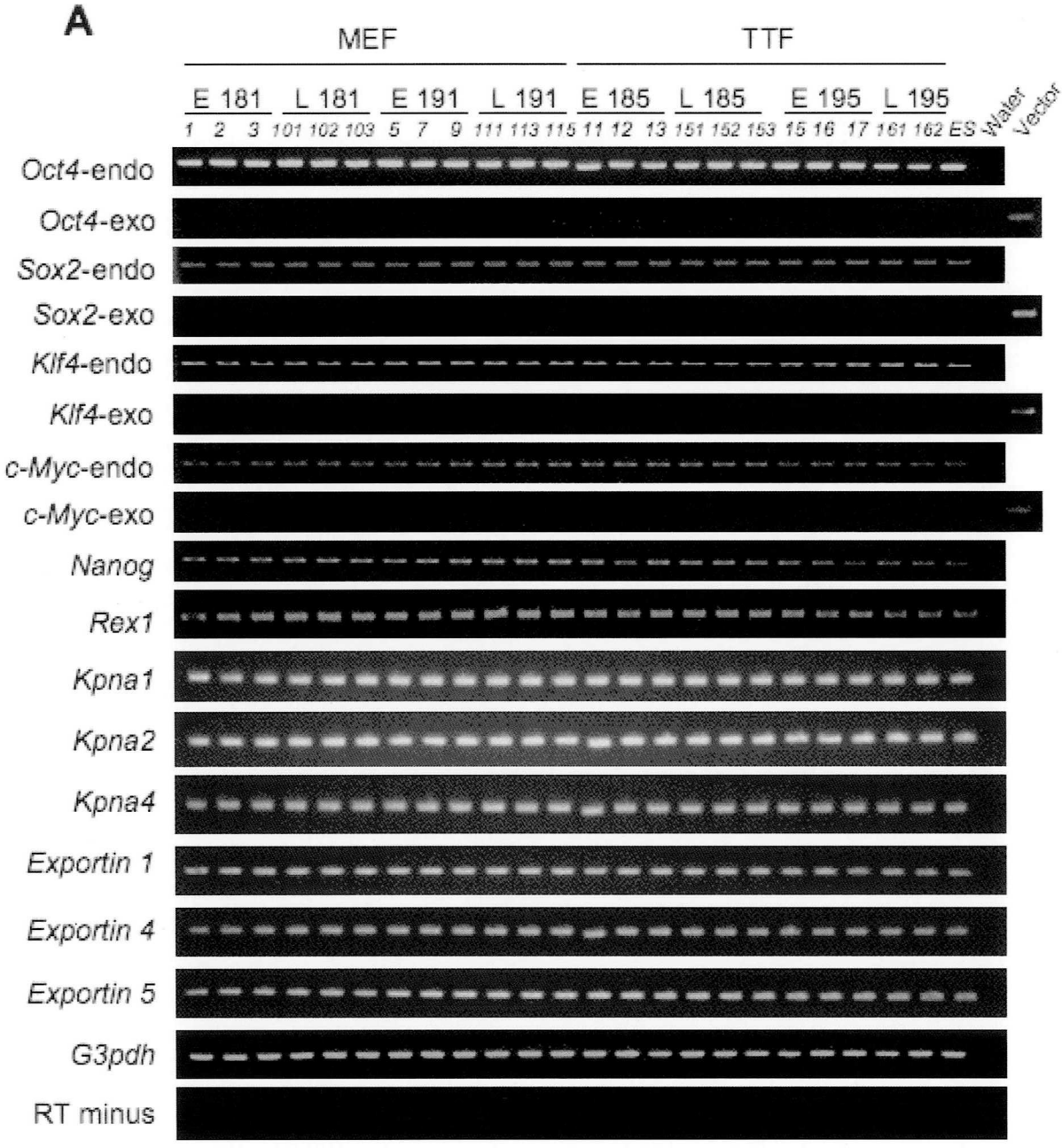
Each iPS clone at passage 16 was seeded into a well of a six-well plate with feeders (day 0) and expanded every two days. Shown are representative iPS (clones 181-1 and 185-151) and ES cells.

Table 2. Doubling times of iPS clones

Clone number	Origin	Timing of retroviral silencing	Doubling times (h)
181-1	MEF	Early	14.3
181-2			13.9
181-3			14.7
181-101		Late	14.3
181-102			14.9
181-103			14.3
191-5		Early	14.5
191-7			14.5
191-9			14.1
191-111		Late	14.3
191-113			14.2
191-115			14.0
185-11	TTF	Early	14.5
185-12			14.4
185-13			14.5
185-151		Late	14.1
185-152			14.4
185-153			14.4
195-15		Early	14.8
195-16			14.4
195-17			14.3
195-161		Late	14.2
195-162			14.7
195-163			18.3
ES cells			14.2

RT-PCR at passage 20 confirmed that the remaining 23 clones expressed pluripotency marker genes at comparable levels to those in ES cells and underwent retroviral silencing (Fig. 4A). Immunofluorescence analysis confirmed that the 23 clones expressed Oct4 and Nanog (Fig. 4B). Furthermore, subclones of the 23 iPS clones at passage 20 were positive for alkaline phosphatase, a marker of ES cells (Fig. 4C). These results show that 12 of 12 early iPS and 11 of 12 late iPS clones at passage 20 are similar to ES cells regarding proliferation efficiency and pluripotency marker expression.

A recent report showed the nuclear import of Oct4 and Sox2 by Kpna1 (also called Importin alpha 5 or Npi1), Kpna2 (also called Importin alpha 1 or Rch1), and Kpna4 (also called Importin alpha 3 or Qip1) [47]. Another group reported that Exportin 4 was one of nuclear transport receptors of Sox2 [48]. Based on these studies, I examined whether there were differences in nuclear transport factors expression among the 23 clones. RT-PCR showed that expression levels of *Kpna1*, *Kpna2*, *Kpna4*, *Exportin 1* (also called *Crm1*), *Exportin 4*, and *Exportin 5* in the 23 clones were comparable to those in ES cells (Fig. 4A). This provides a possibility that the efficiency of nuclear import of Oct4 and Sox2 in my early and late iPS clones might be similar to those in ES cells.


A
MEF
TTF
E 181
L 181
E 191
L 191
E 185
L 185
E 195
L 195
1 2 3 101 102 103 5 7 9 111 113 115 11 12 13 151 152 153 15 16 17 161 162 ES
Water
Vector
Oct4-endo
Oct4-exo
Sox2-endo
Sox2-exo
Klf4-endo
Klf4-exo
c-Myc-endo
c-Myc-exo
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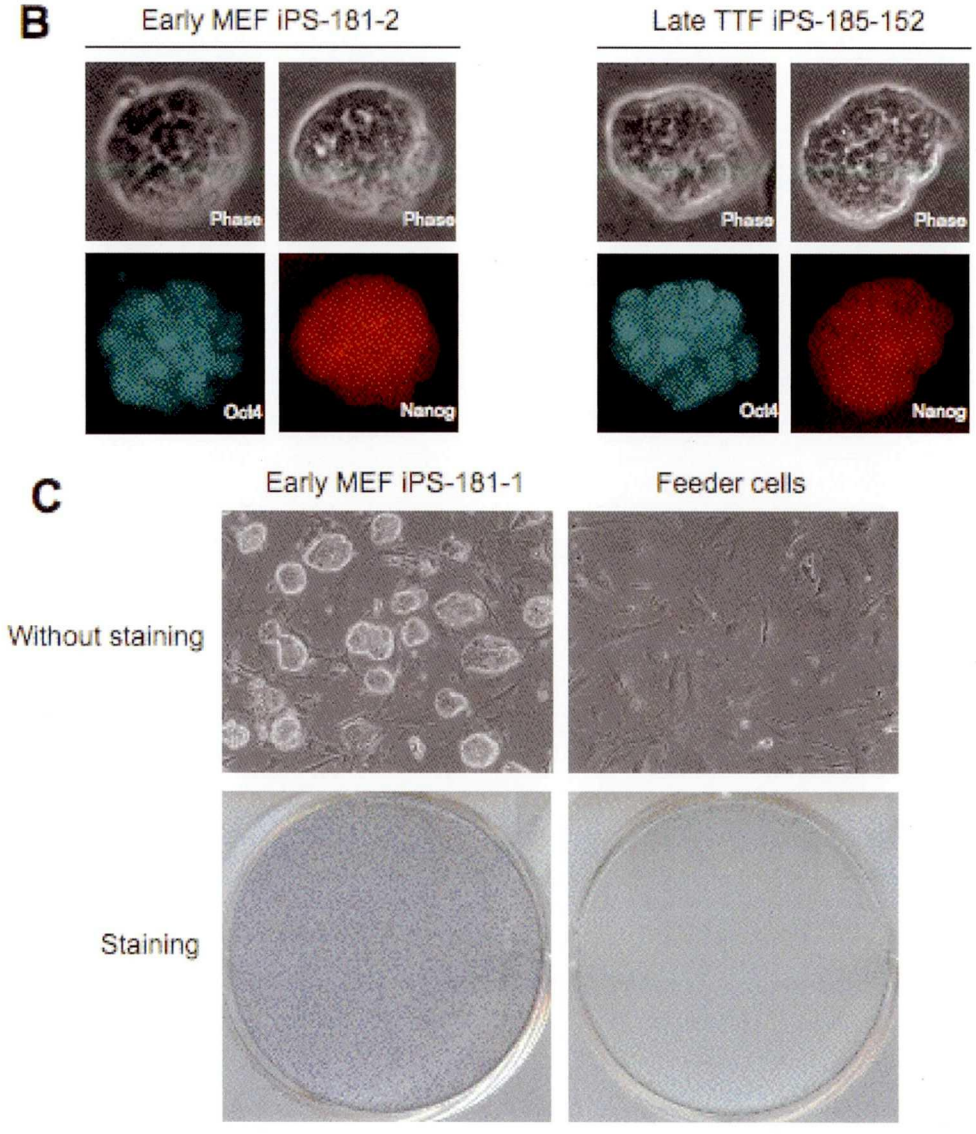


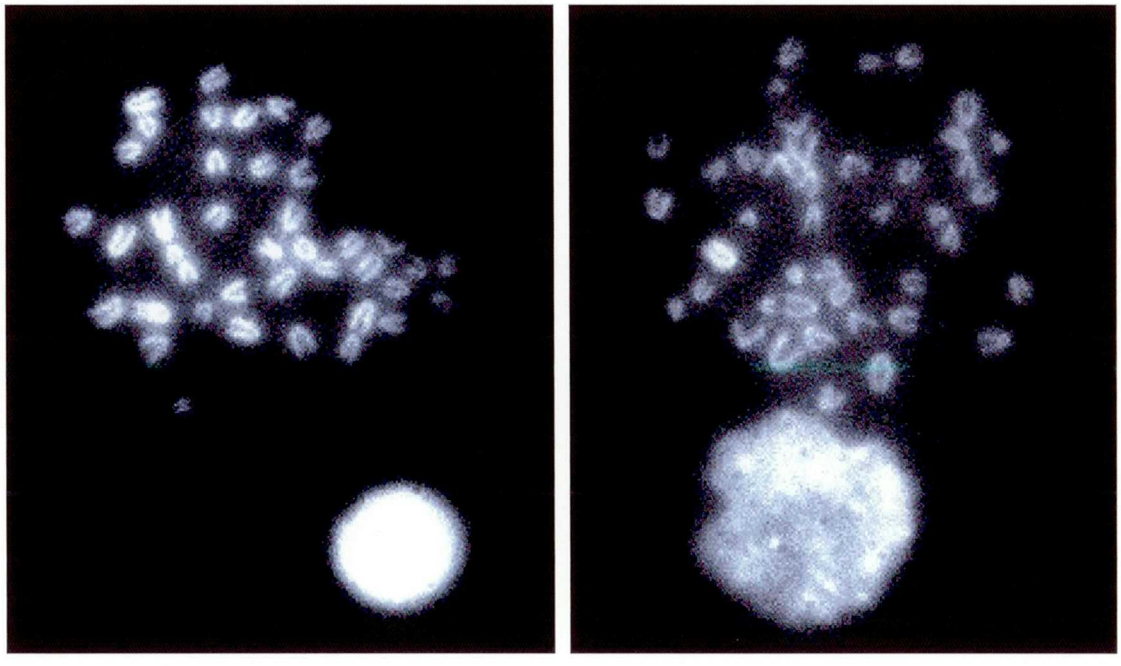
Figure 4. ES cell marker expression of iPS clones at passage 20.

(A) RT-PCR. Total RNA was extracted from six early MEF iPS (clones 181-1, -2, and -3; 191-5, -7, and -9), six late MEF iPS (clones 181-101, -102, and -103; 191-111, -113, and -115), six early TTF iPS (clones 185-11, -12, and -13; 195-15, -16, and -17), five late TTF iPS (clones 185-151, -152, and -153; 195-161 and -162), and ES cells. The expression of ES cell marker genes (*Oct4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, and *Rex1*), retroviral transgenes, and nuclear transport factors (*Kpna* and *Exportin*) was examined. (B) Immunofluorescence. The expression of Oct4 and Nanog was analyzed. Phase and fluorescence microscopy images of representative iPS (clones 181-2 and 185-152) are shown. (C) Alkaline phophatase staining. iPS clones ($1x10^5$ cells) at passage 20 were seeded into a well of a six-well plate with feeders. Two days later, the cells were photographed (top) and stained for alkaline phophatase (bottom). A representative iPS clone (early MEF iPS-181-1) and feeder cells are shown

Chromosomal stability of iPS clones

I next examined the chromosomal stability of the 12 early iPS and 11 late iPS clones by karyotyping (Fig. 5A, Table 3, and Supplementary Fig. 1). The results at passage 3 showed that there was no significant difference in the ratio of cells with a normal karyotype between early and late MEF iPS clones ($p = 0.19$, Fig. 5B) and between early and late TTF iPS clones ($p = 0.67$, Fig. 5D). In contrast, at passage 20, the ratio (18-36%) observed in late MEF iPS clones was lower than that (45-86%) in early MEF iPS clones ($p < 0.01$, Fig. 5C). Similarly, at passage 20, the ratio (18-55%) observed in late TTF iPS clones was lower than that (55-100%) in early TTF iPS clones ($p < 0.01$, Fig. 5E). These data demonstrate that early iPS clones are more stable than late iPS clones.

A



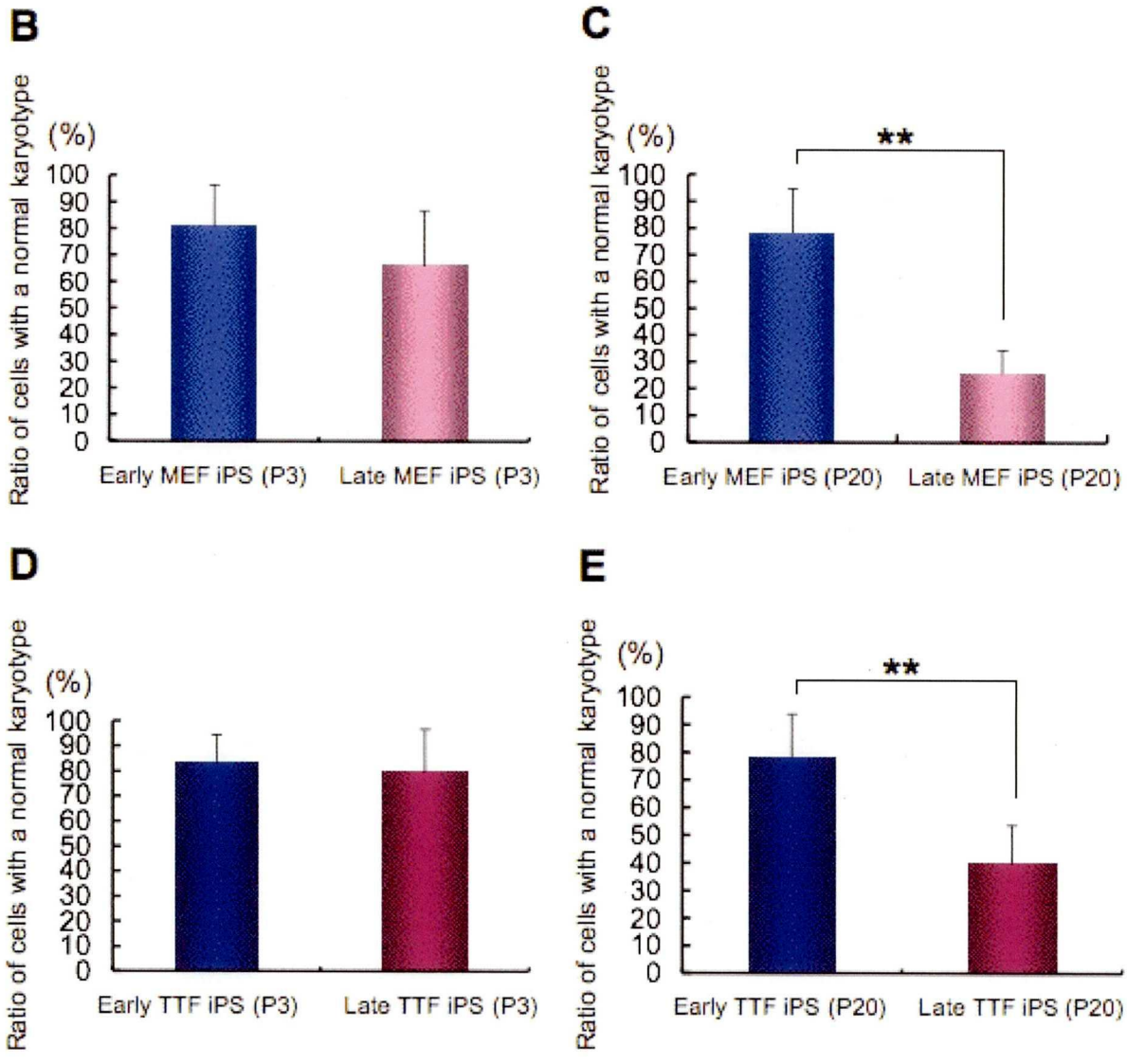


Figure 5. Chromosomal stability of iPS cells.

(A) Normal karyotype of 40 chromosomes (left) and abnormality of 42 chromosomes (right) are shown. (B–D) The ratio of iPS cells with a normal karyotype was calculated (error bars indicate s.d.). (B) Six early MEF iPS and six late MEF iPS clones at passage 3 were examined ($p = 0.19$). (C) Six early MEF iPS and six late MEF iPS clones at passage 20 were examined ($^{**}p < 0.01$). (D) Six early TTF iPS and five late TTF iPS clones at passage 3 were examined ($p = 0.67$). (E) Six early TTF iPS and five late TTF iPS clones at passage 20 were examined ($^{**}p < 0.01$).

Table 3. Data for karyotyping

Clone number	Origin	Timing of retroviral silencing	% Ratio of cells with a normal karyotype (P3)	% Ratio of cells with a normal karyotype (P20)
181-1	MEF	Early	100	86
181-2			86	86
181-3			86	86
181-101		Late	86	27
181-102			45	36
181-103			71	18
191-5		Early	71	71
191-7			86	86
191-9			55	45
191-111		Late	86	18
191-113			71	36
191-115			36	18
185-11	TTF	Early	100	100
185-12			86	71
185-13			86	86
185-151		Late	86	45
185-152			100	55
185-153			86	18
195-15		Early	86	86
195-16			71	55
195-17			71	71
195-161		Late	55	45
195-162			71	36

ES cells (P26)	86

Pluripotency of iPS clones

I next evaluated the developmental potential of early iPS clones. I used 10 of the 12 early iPS clones at passage 20, but excluded clones 191-9 and 195-16, because the ratio of cells with a normal karyotype was less than 60% in these clones (Table 3). We injected early iPS cells into eight-cell-stage embryos or blastocysts derived from ICR mice. We cultured the eight-cell-stage embryos until the blastocyst stage and then transplanted the blastocysts into pseudo-pregnant ICR females. We obtained chimeric mice from all of the early iPS clones injected (Fig. 6A and Table 4). Polymerase chain reaction (PCR) analysis confirmed viral integration in these chimeric mice (Fig. 6B), indicating iPS cell contribution.

I crossed male chimeras derived from all 10 different iPS clones with ICR females. I obtained viable offspring with black or agouti coat color from chimeras derived from four of five early MEF iPS clones and one of five early TTF iPS clones (Fig. 7A and Table 4). PCR analysis detected transgene integration in the offspring (Fig. 7B). These results suggest that MEFs are more amenable to full reprogramming than adult TTFs. Taken together, my results indicate that stable and germline transmittable iPS cells can be efficiently generated by establishing ES cell-like colonies undergoing retroviral silencing at earlier time points.

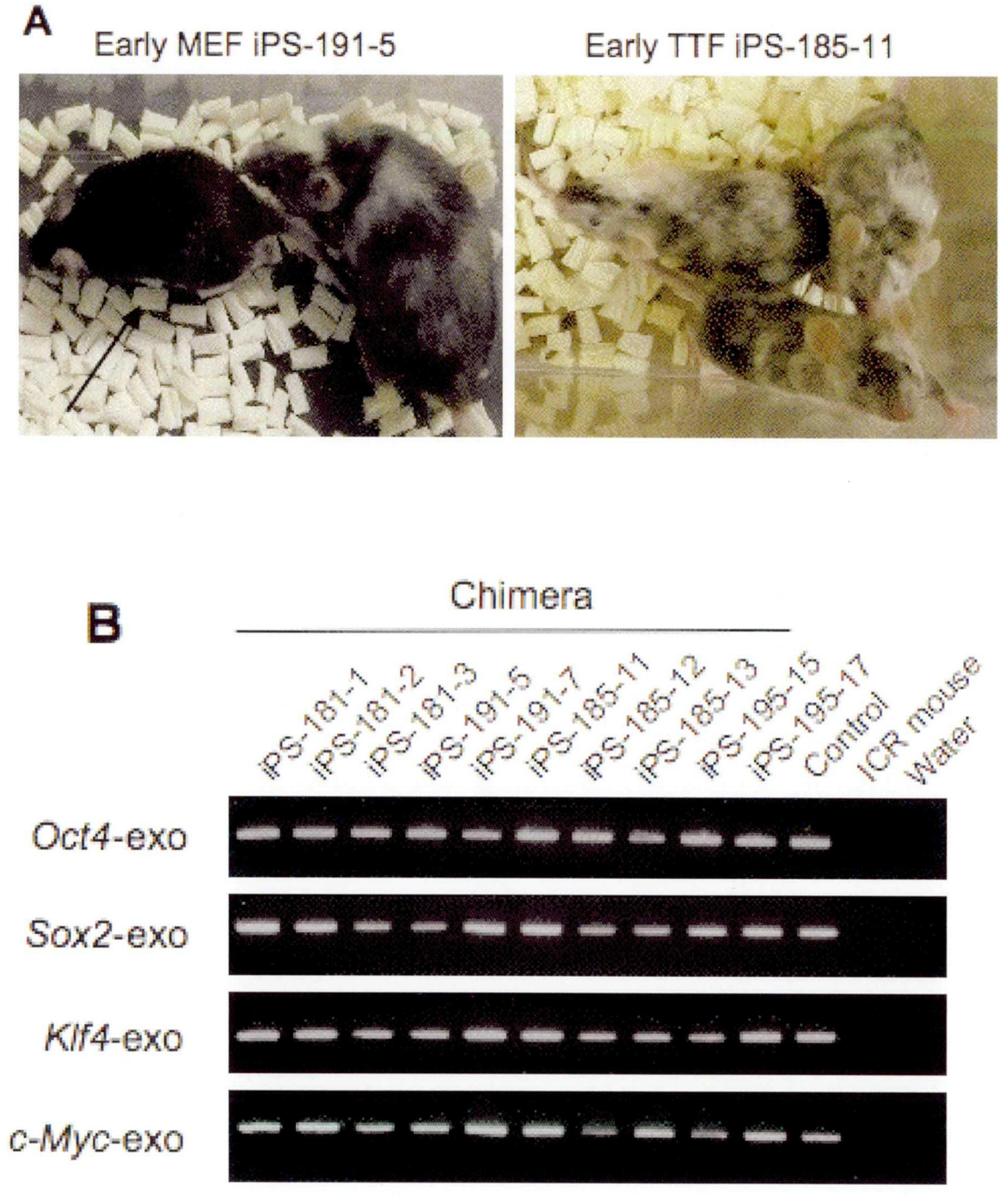


Figure 6. iPS-derived chimeric mice.

(A) Chimeras generated from clones early MEF iPS-191-5 and early TTF iPS-185-11 are shown. The arrow indicates a chimera with chimerism of greater than 90% (left panel), which was produced by eight-cell-stage embryo injection. (B) PCR genotyping. Genomic DNA was extracted from chimeras derived from iPS cells (clones 181-1, -2, and -3; 191-5 and -7; 185-11, -12, and -13; 195-15 and -17) and an ICR mouse. DNA of the chimera derived from iPS-102A5 in our previous report was used as a control. Retroviral integration of the four factors was analyzed.

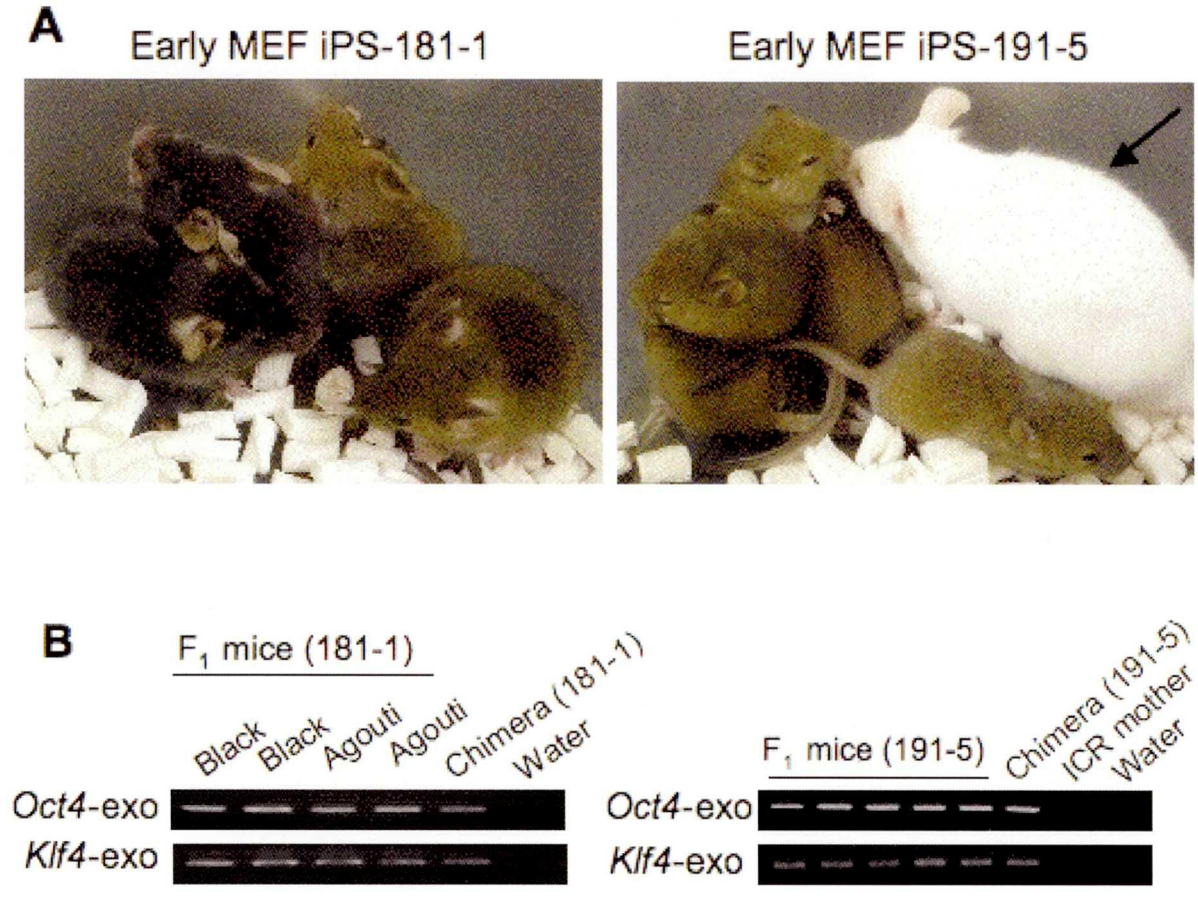


Figure 7. Germline transmittable iPS cells.

(A) Coat colors of F_1 mice born from iPS-181-1- and iPS-191-5-derived chimeras crossed with wild-type ICR females. The arrow indicates the ICR mother (right panel).

(B) Genotyping. DNA was isolated from F_1 mice (two black and two agouti mice derived from iPS-181-1 and five agouti mice derived from iPS-191-5) and the ICR mother. Viral copies of *Oct4* and *Klf4* were examined.

Table 4. Data for chimera generations

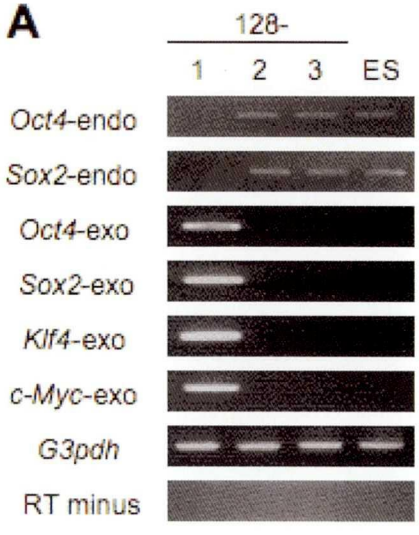
Clone number	Origin	Timing of retroviral silencing	Injected blastocysts	Injected 8-cell-stage embryos	Births	Adult chimeras	Chimerism		Germline transmission*
							10-90%	> 90%	
181-1	MEF	Early	60		33	14	14	0	62/64
				20	6	1	1	0	10/15
181-2	MEF	Early	60		29	12	12	0	25/51
				20	2	1	0	1	N.D.
181-3	MEF	Early	60		20	1	1	0	N.D.
				20	5	0	0	0	0
191-5	MEF	Early	60		31	12	12	0	12/61
				20	8	7	5	2	30/60
191-7	MEF	Early	60		27	11	11	0	19/87
				20	0	0	0	0	0
185-11	TTF	Early	60		37	13	13	0	2/113
				20	2	0	0	0	0
185-12	TTF	Early	60		22	5	5	0	N.D.
				20	11	7	7	0	N.D.
185-13	TTF	Early	60		27	9	9	0	N.D.
				20	2	1	1	0	N.D.
195-15	TTF	Early	60		25	5	5	0	N.D.
				20	0	0	0	0	0
195-17	TTF	Early	60		35	2	2	0	N.D.
				20	2	0	0	0	0

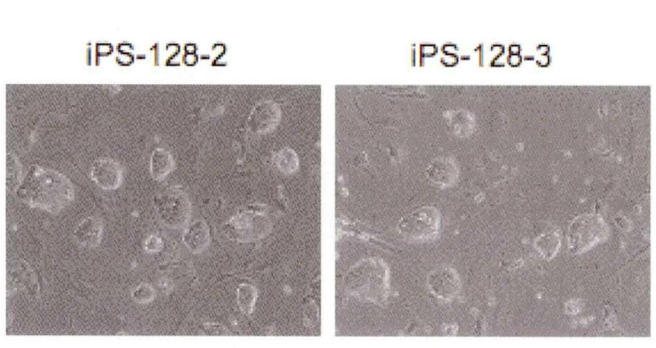
* iPS-derived F_1 mice/total offspring; N.D., not determined.

Timing of retroviral silencing of individual factors

I next analyzed the sequence of retroviral silencing of individual genes in MEF iPS cell derivation. First, I examined whether primary colonies express endogenous *Oct4*, as described by other reports [20, 21]. On day 14, I picked three ES cell-like colonies (clones 128-1, -2, and -3) produced by the four retroviruses. I used half of one colony for RT-PCR and expanded the remaining half. RT-PCR at passage 0 showed that two of the three clones

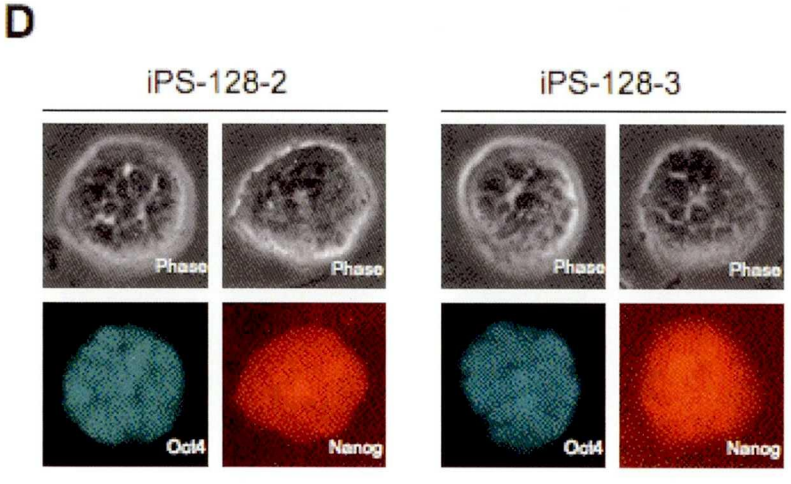
expressed endogenous *Oct4* at a comparable level to that in ES cells (Fig. 8A). At passage 10, the two clones (128-2 and -3) were similar to ES cells in terms of morphology, proliferation efficiency, ES marker expression, and karyotype (Fig. 8B–D). Furthermore, We generated viable F_1 mice using the two clones (Fig. 8E). These data indicate that endogenous *Oct4* activation can be used as a marker of germline transmittable iPS cells, which is consistent with a previous report [21].





C

Clone number	Origin	Doubling times (h)	% Ratio of cells with a normal karyotype (P10)
128-2	MEF	14.7	86
128-3		14.1	86
ES (P26)		14.2	86



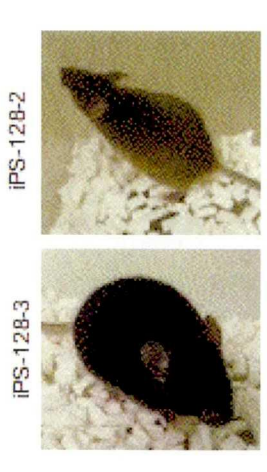
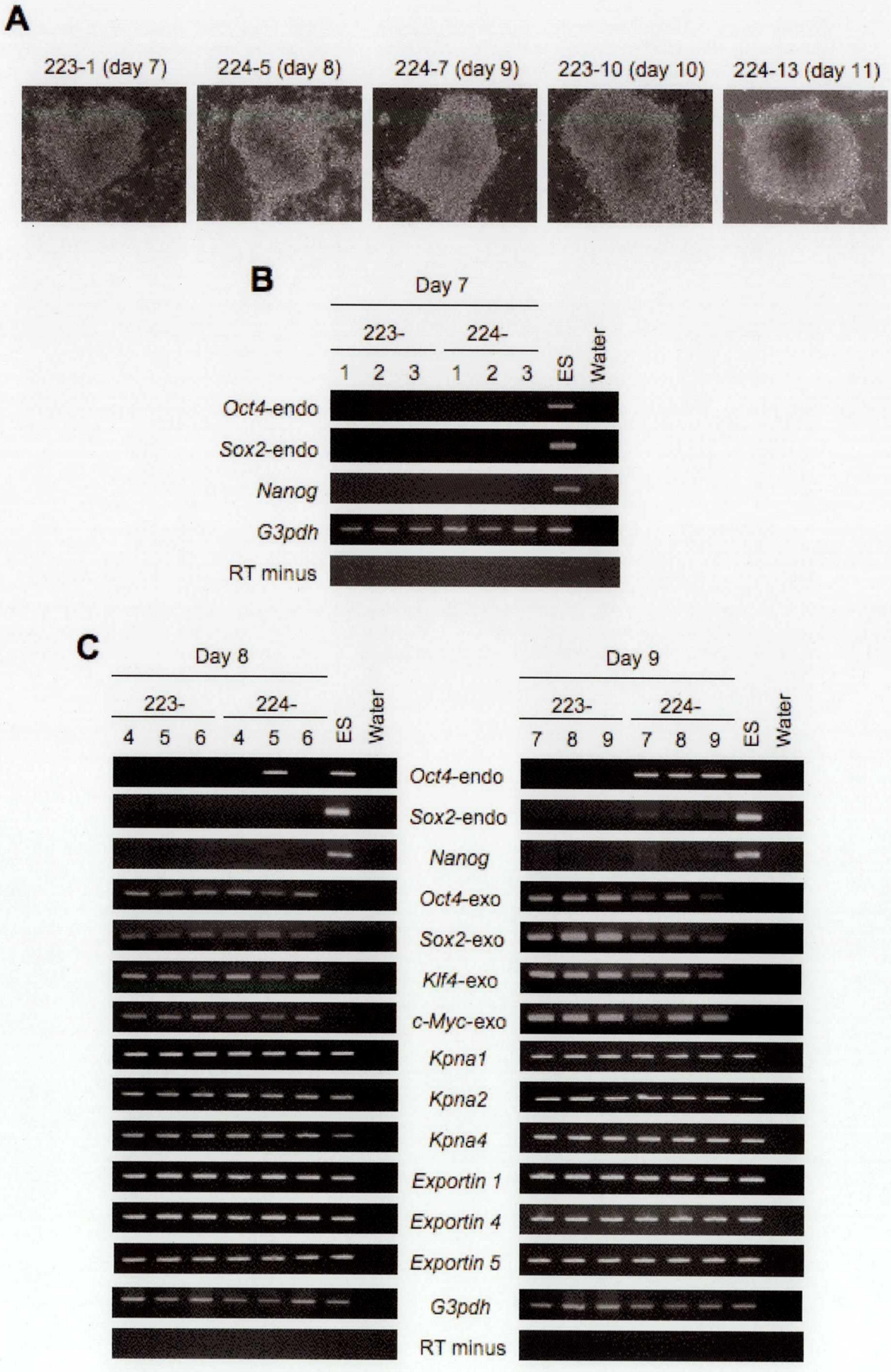


Figure 8. Germline transmittable iPS cells selected by endogenous *Oct4* expression.
(A) RT-PCR. Total RNA was extracted from primary iPS colonies (128-1, -2, and -3) and ES cells. (B) Morphology of iPS clones (128-2 and -3) at passage 10. (C) Proliferation efficiency and ratio of cells with a normal karyotye. (D) Immunofluorescence. The expressions of Oct4 and Nanog were examined. (E) F_1 mice derived from iPS-128-2 and -3.

I then picked six ES cell-like colonies on each day from day 7 to day 11 (Fig. 9A). On day 7, RT-PCR at passage 0 showed that endogenous *Oct4* was not detectable in the six colonies (Fig. 9B). On days 8 and 9, among the 12 colonies, four colonies (224-5, -7, -8, and -9) expressed endogenous *Oct4*, whereas the other eight colonies did not, and none of the colonies silenced all four retroviral transgenes (Fig. 9C). On days 10 and 11, among the 12 colonies, four colonies (223-10 and -15; 224-13 and -15) lost the expression of all of the transgenes, while six colonies (223-11, -12, -13, and -14; 224-10 and -11) still expressed all of the transgenes, and these 10 colonies expressed endogenous *Oct4* (Fig. 9D). In addition, the other two colonies (224-12 and -14) did not express endogenous *Oct4*, and still expressed all of the transgenes. These results imply that retroviral silencing of all of the factors proceeds almost simultaneously during MEF reprogramming in colonies undergoing silencing at early time points.

I also examined expression levels of *Kpna1*, *Kpna2*, *Kpna4*, *Exportin 1*, *Exportin 4*, and *Exportin 5* in the colonies picked on days 8-11. All of the colonies expressed these nuclear transport factors at levels similar to those in ES cells (Fig. 9C and D). My data suggest that variations in Oct4, Sox2, and Nanog expression and retroviral silencing of the different colonies may not be caused by abnormality of nuclear transport signals. Understanding of how retroviral genes are almost simultaneously silenced is valuable to the field, and therefore I will continue to analyze it in a further study.


A
223-1 (day 7)
224-5 (day 8)
224-7 (day 9)
223-10 (day 10)
224-13 (day 11)
B
Day 7
223-
224-
1 2 3 1 2 3
ES
Water
Oct4-endo
Sox2-endo
Nanog
G3pdh
RT minus
C
Day 8
Day 9
223-
224-
4 5 6 4 5 6
7 8 9 7 8 9
ES
Water
Oct4-endo
Sox2-endo
Nanog
Oct4-exo
Sox2-exo
Klf4-exo
c-Myc-exo
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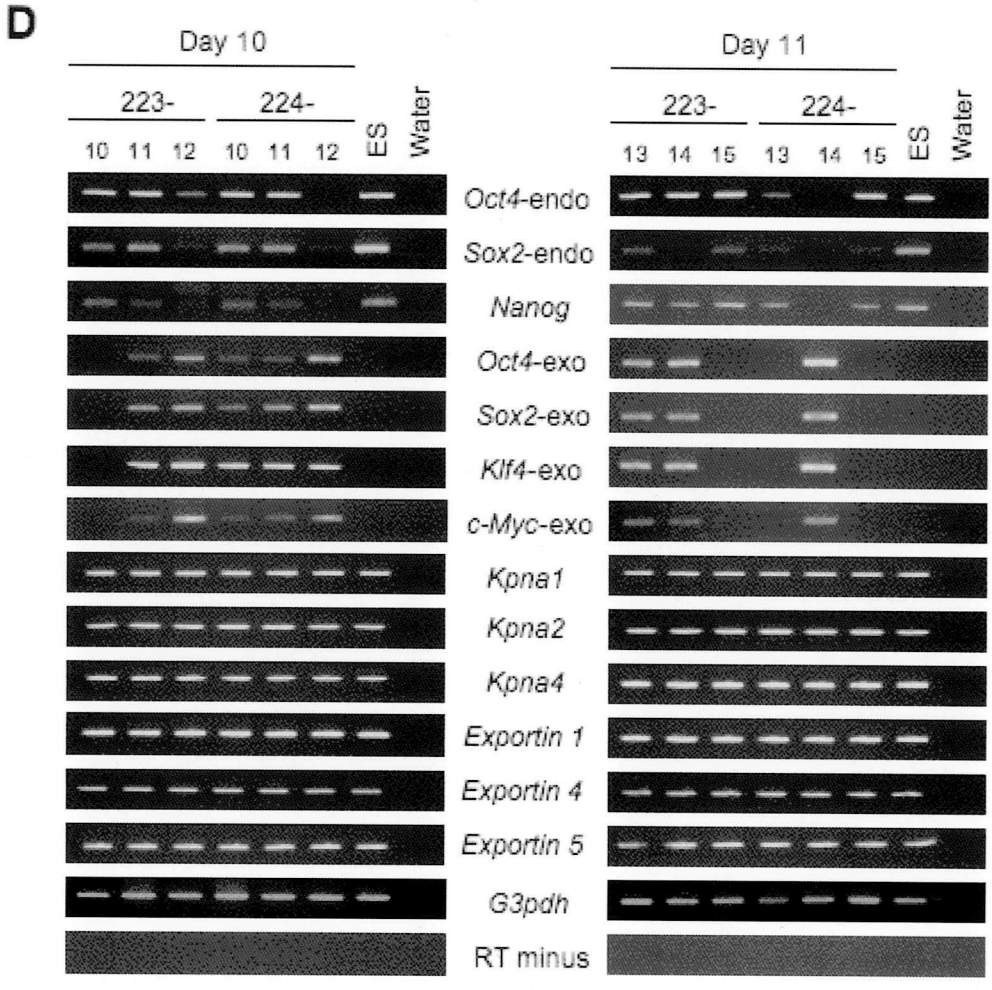

Figure 9. Retroviral silencing of individual factors and gene expression.

(A) Morphology of ES cell-like colonies generated from MEFs on days 7–11.

(B–D) RT-PCR. Total RNA was isolated from primary ES cell-like colonies (passage 0) and ES cells. The expression of ES cell marker genes, retroviral transgenes, and nuclear transport factors was analyzed.

Discussion

I demonstrate here that early iPS cells produced from both MEFs and adult TTFs are more stable than late iPS cells (Fig.10), when retroviral silencing is used as a criterion of iPS cells. Most of the early iPS clones maintained ES cell-like morphology and a normal karyotype at high rate during 20 passages. In addition, these lines expressed pluripotency marker genes at levels comparable to those in ES cells and proliferated efficiently like ES cells. Furthermore, I successfully generated adult chimeras from all early iPS clones injected. In contrast, late iPS clones tended to lose their ES cell-like morphology and chromosomal stability by passage 20. These data suggest that early completion of reprogramming is a crucial factor in stable iPS cell derivation, and that long-term expression of the retroviral transgenes during reprogramming might cause the resulting iPS cells to be unstable, at least in mouse. Although I used retroviral silencing as a pluripotency marker in this study, selection for Oct4, Nanog, or Sox2 protein activation can also be used to establish superior iPS cells. It is currently known that the expression of these endogenous marker genes correlates with retroviral silencing in primary ES-like colonies [40, 41]. Therefore, when the selection markers are utilized, stable iPS cells may be obtained by establishing ES-like colonies expressing the markers at earlier time points. It is important to examine the stability of mouse and human iPS cells generated with other reprogramming methods that do not alter the host genome, such as adenoviruses [34, 35], plasmid vectors [36], and recombinant proteins [37, 38].

I found that my early MEF iPS clones showed germline competency at an efficiency of 80% (four of five clones tested, Table 4). This implies that early completion of reprogramming may be an important determinant of germline transmission of MEF iPS cells.

It is possible that the appropriate culture conditions, which we have previously reported [42], may allow for the efficient generation of germline transmittable MEF iPS cells. In the case of TTF iPS cells, however, only one clone showed germline contribution and the number of iPS-derived offspring was very low (Table 4). Furthermore, I did not observe high chimerism in TTF iPS-derived chimeric mice, whereas chimerism of greater than 90% was seen in MEF iPS-derived chimeras (Fig. 6A and Table 4). This suggests that the efficiency of generating germline transmittable iPS cells may be influenced by donor cell type and that undefined factors, which diminish the differentiation ability of pluripotent cells, may exist in TTFs or TTF iPS cells. Alternatively, the genetic background of donor cells or reprogramming methods may affect the characteristics of iPS cells, given that the developmental potential of adult somatic cell-derived iPS cells has been reported to be comparable to that of ES cells [34, 49, 50].

It is believed that germline transmission is one of the most stringent tests for the pluripotency of mouse iPS cells. Hence, I evaluated my iPS clones by germline transmission in addition to the criteria of morphology, proliferation efficiency, ES marker expression, and karyotype. My results indicated that there was a variation in germline competence of the 10 iPS clones (Table 4), although I did not observe remarkable difference in quality among these clones by *in vitro* criteria. This means that it is crucial to evaluate mouse iPS clones by germline transmission in order to examine whether produced clones are fully reprogrammed. Furthermore, the criterion of retroviral silencing might have a limitation in full reprogramming of adult TTFs. In the case of human iPS cells, germline transmission cannot be tested because of ethical problems, and therefore it is hard to clearly demonstrate full reprogramming of human somatic cells. However, when human iPS cells

are used for *in vitro* applications, such as drug screening and disease modeling, it is sufficient for the resulting cells to self-renew and produce useful progeny [51]. Thus, it should be noted that evaluation methods for iPS cells may be flexibly considered according to the purpose of study.

Although it remains to be determined whether my early MEF and TTF iPS cells can produce full-term embryos and mice by tetraploid complementation, as reported by others [52-54], and whether established early iPS cells can maintain pluripotency in prolonged culture of more than 20 passages, this study develops a method for inducing stable and germline transmittable iPS cells, and will be an important step towards optimizing the technology for efficient generation of iPS cells that can be available for *in vitro* and *in vivo* medical applications.

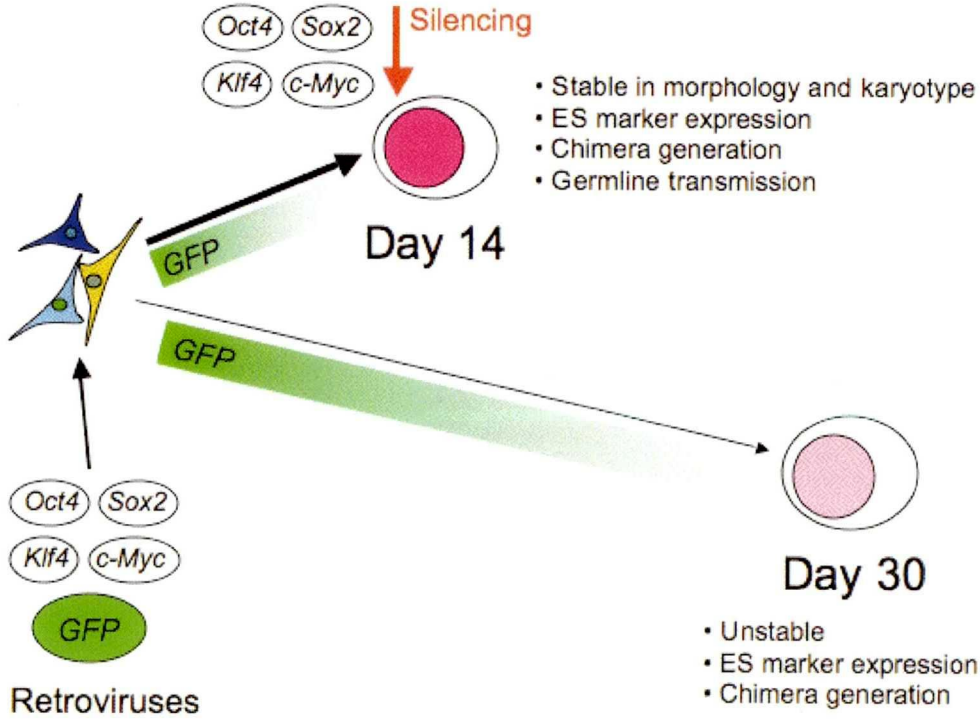

Figure 10. Correlation of the timing of retroviral silencing with the quality of iPS cells.
The lack of exogenous GFP expression reflects the silencing of retroviral Oct4, Sox2, Klf4, and c-Myc. Retroviral silencing on day 14 allows for the efficient selection of stable iPS cells.

Acknowledgments

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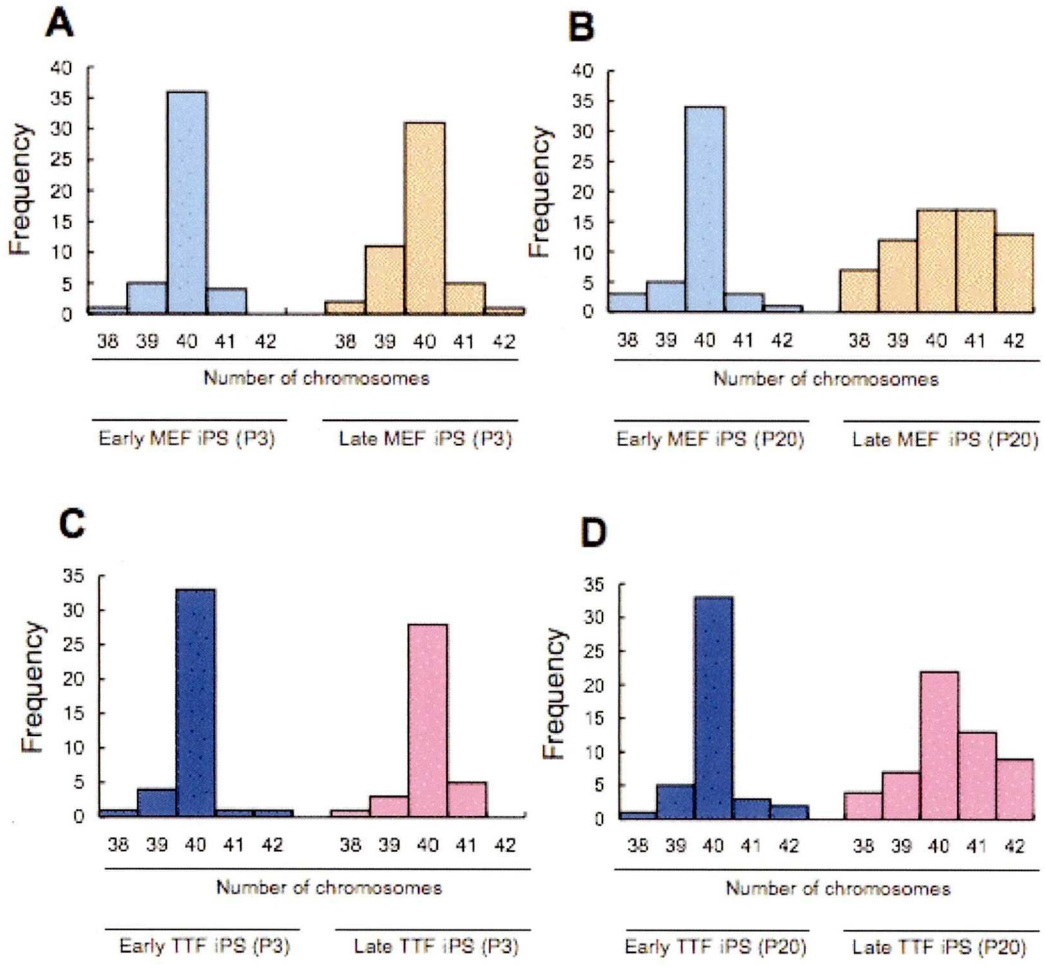
Supplementary data



Supplementary Figure 1. Number of chromosomes in iPS clones.

(A-D) Normal karyotype of 40 chromosomes and abnormality (38, 39, 41, or 42 chromosomes) were detected.