Hypoxia Enhances the Generation of induced Pluripotent Stem Cells

Yoshinori Yoshida¹*, Kazutoshi Takahashi¹, Keisuke Okita¹, Tomoko Ichisaka², Shinya Yamanaka¹,³,²,⁴*

¹Center for iPS Cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto 606-8507, Japan.
²Yamanaka iPS Cell Special Project, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan.
³Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan.
⁴Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94158, USA.

*Correspondence: yoshinori@frontier.kyoto-u.ac.jp, yamanaka@frontier.kyoto-u.ac.jp

Mouse and human somatic cells can be reprogrammed to induced pluripotent stem cells (iPS Cs) by the transduction of four transcription factors, Oct 3/4, Sox2, Klf4, and c-Myc (Maherali et al., 2007; Meissner et all., 2007; Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Patient or disease-specific human iPSCs could be used
for studying pathogenesis, or potentially also to treat patients suffering from incurable diseases by transplanting the regenerated grafts derived from their own cells. However, the low induction efficiency and high tumorigenesis rate due to the use of proto-oncogenes, such as c-Myc, continue to hinder the clinical application of iPS technology. Many efforts have been made to find other factors or small molecules that facilitate the reprogramming process (Huangfu et al., 2008; Shi et al., 2008b). In this study we show that conducting reprogramming in hypoxic conditions results in improved efficiency for both mouse and human cells.

Somatic stem cells reside in specific microenvironments, called niches, and environmental changes, such as stromal cell contacts, extracellular matrix proteins, temperature, and O₂ tension, have a great influence on stem cell function and differentiation. Notably, low O₂ tension promotes the survival of neural crest cells and hematopoietic stem cells and prevents differentiation of human ES cells (Danet et al., 2003; Ezashi et al., 2005; Morrison et al., 2000). Moreover, mammalian embryonic epiblasts reside in a physiologically hypoxic environment. These observations led us to the hypothesis that hypoxic conditions might promote the reprogramming process and thus iPS cell generation.

To quantify the effect of hypoxia on iPS cell generation, comparison experiments were performed on mouse embryonic fibroblasts (MEFs) carrying the Nanog-GFP-Ires-Puro’ cassette
Four or three transcription factors (Oct3/4, Sox2, Klf4, +/- c-Myc) were introduced into MEFs using retroviral vectors. Four days posttransduction, the cells were trypsinized and seeded onto the feeder layer of mitomycin C-treated STO cells. The cells were cultivated under 21%, 5%, or 1% O₂ from day 5 to day 14 posttransduction. GFP+ iPS cell colonies could be first detected on days 10-14 after viral transduction, and we counted the number of GFP-positive colonies on days 21 and 28 posttransduction. Under 5% O₂, the GFP-positive colonies derived from four-factor transduced MEFs increased 7.4-fold on day 21 and 3.1-fold on day 28, and those from three-factor transduced MEFs increased 20-fold on day 21 and 7.6-fold on day 28 under 5% O₂ (Figure 1-A-a, b). Moreover, hypoxic treatment increased the percentage of GFP-positive colonies in total colonies from four- or three-factor transduced MEFs (Figure 1-A-c, d). The GFP-positive colonies derived after hypoxic treatment was comparable in morphology and size to those derived under normoxic conditions (Figure S1). Alkaline phosphatase staining showed that cultivation under 5% O₂ increased the number of colonies with a positive alkaline phosphatase activity (Figure S2). We also examined whether GFP-positive cells were detected more quickly under hypoxic conditions. The four-factor transduced MEFs were cultivated under 21% O₂ or under 5% O₂ with or without VPA from day 5 to day 9 posttransduction, and were subjected to flow cytometric analysis on day 9. Retroviral
expression of four factors induced 0.01% of the cells to become GFP-positive on day 9 posttransduction. Treating the four-factor transduced MEFs for four days with hypoxia or with VPA increased the percentage of GFP-positive cells to 0.40% and 0.48%, respectively. Moreover, co-treatment with hypoxia and VPA increased the percentage of GFP-positive cells to 2.28%. These data suggest that GFP-positive cells can be detected earlier and that the effect of hypoxic culture synergises with VPA (Figure 1-B-a, b, c, d).

While neural stem cells which express SOX2 endogenously can be reprogrammed to iPS cells with the transduction of Oct3/4 and Klf4 (Kim et al., 2008), the reprogramming of MEFs to iPS cells rarely occurs with two transcription factors of Oct3/4 and Klf4. Recently, small-molecule compounds have been reported to enable the reprogramming of Oct3/4 and Klf4-transduced MEFs to iPS cells (Shi et al., 2008a). We examined whether hypoxic conditions could enhance MEFs to be reprogrammed to iPS cells with Oct3/4 and Klf4 transduction. Figure 1-A-e shows an increased efficiency of the iPS cell generation derived from MEFs with Oct3/4 and Klf4 (MEF-2F-iPS) under 5% O2 in comparison to 21% O2.

To further evaluate the pluripotency of the iPS cells generated with hypoxic treatment, we randomly picked up and established multiple iPS cell lines from two-, three-, and four-factor infected MEFs. These iPS cells exhibited typical ES cell morphology. We examined the
karyotype of iPS cell lines derived after hypoxic treatment (521AH5-1 and 527CH5-1), and these cell lines showed normal karyotypes (Figure S3). We investigated the mRNA expression of pluripotency-related genes in the iPS cells generated after hypoxic treatments. The mRNA expression patterns of these iPS cells were comparable to those of ES cells (Figure S4). When injected into nude mice subcutaneously, all of the established iPS cell lines gave rise to teratomas with histologic evidence of cells differentiating into all three germ layers (Figure S5-a, b, c, d, e). Moreover, MEF-2F-iPS cells derived under hypoxic conditions contributed to the formation of somatic tissue in chimeric mice (Figure 1-C-a, b), but we have not yet obtained germline transmission with these mice, so the extent of reprogramming is not entirely clear. Previous studies have shown that iPSCs generated with the same 3 or 4 factors are capable of germline transmission (Nakagawa et al., 2008; Okita et al., 2007).

Recently, it was reported that iPS cells could be established by other methods than retroviruses or lentiviruses. We reported that transient transfection of expression plasmid vectors of four reprogramming factors could reprogram MEFs to iPS cells (Okita et al., 2008). It was also reported that MEFs could be reprogrammed by transcription factors delivered by piggyBac (PB) transposition system (Kaji et al., 2009; Woltjen et al., 2009). The PB insertions can be removed from established iPS cells. These methods minimize the potential for
insertional mutagenesis. We examined whether hypoxia could improve the efficiency of iPS cell generation with plasmid vectors and with PB transposition system. Figure 1-D shows that hypoxic cultivation significantly increased the number of GFP-positive colonies with transient transfection of plasmid vectors, and Figure 1-E shows that hypoxic treatment for 5 and 10 days increased the number of GFP-positive colonies with the piggyBac transposition system by 2.9-fold and 4.0-fold, respectively. These data suggest that hypoxia can increase the efficiency of iPS cell generation by non-viral vectors such as plasmid expression vectors or the piggyBac transposition system.

We next examined the effect of hypoxic culture on proliferation, survival, and gene expression. Flow cytometric analysis using annexin V demonstrated that hypoxic culture had no protective effect on mouse ES cells or on four-factor transduced MEFs (Figure S6). Furthermore, hypoxic cultivation showed no effect on proliferation of mouse ES cells (Figure S7). Although hypoxic incubation from day 1 to day 4 posttransduction had no significant effect on proliferation of mock transduced MEFs, it had significant effect on four-factor transduced MEFs (Figure S8). To investigate the expression profile of cells in reprogramming process, we performed microarray analysis and quantitative real-time RT-PCR. We examined the expression profile of MEFs cultivated under hypoxic and normoxic conditions for 10 days. Microarray analysis shows
that 57.2% of ES cell-specific genes were up-regulated and 67.5% of MEF-specific genes were down-regulated in the MEFs cultivated under 5%O\textsubscript{2} (Figure S9-A, B). In Figure S9-C and D, microarray analysis of four-factor transduced MEFs cultivated under hypoxic and normoxic conditions from day 1 to day 4 showed that 73.2% of ES cell-specific genes (765 genes out of 1045 total genes) were up-regulated and 85.8% of MEF-specific genes (980 genes out of 1142 total genes) were down-regulated in the cells treated with hypoxia. Moreover, quantitative real-time RT-PCR analysis demonstrated that expression of endogenous Oct3/4 and Nanog increased 3.4-fold and 2.1-fold respectively in four-factor transduced MEFs after hypoxic treatment of three days (Figure S9-E, F).

To rule out the possibility that hypoxia enhances iPS cell generation by stimulating STO cells, we examined growth situation of iPS cells under hypoxic cultivation without the feeder layer of STO cells. Figure S10 shows that cultivation under 5%O\textsubscript{2} increased the number of GFP-positive colonies, suggesting that hypoxic enhancement of reprogramming was not mediated by STO cells.

We next examined whether or not the exposure to hypoxia increases the efficiency of iPS cell generation from human somatic cells. The four transcription factors were introduced into adult human dermal fibroblasts (HDFs) by retroviral vectors. At six days posttransduction,
the cells were trypsinized and seeded onto the feeder layer of mitomycin C-treated STO cells. The cells were cultivated under 5% O₂ for 7 (1w), 14 (2w), 21 (3w), or 33 days (Long), and the number of human ES-like colonies was counted on day 24, 32, and 40 posttransduction (Figure 2-A). Figure 2-B shows that 14-day and 21-day hypoxic cultivation increased the efficiency of iPS cell generation by 4.2-fold and 3.6-fold on day 24, by 2.8-fold and 3.0-fold on day 32, and by 2.6-fold and 2.5-fold on day 40, respectively. We randomly selected and established several clones of human iPS cells derived under hypoxic conditions. All of the human iPS cell lines had a typical ES cell morphology and were strongly positive for alkaline phosphatase while also expressing pluripotent-related gene markers (Figure 2-C-a, b, 2-D). Moreover, immunocytological staining showed that all of the cell lines expressed SSEA3, SSEA4, and Nanog (Figure 2-C-c, d, e).

To investigate the differentiation ability of the human iPS cells derived under hypoxic conditions, we used floating cultivation to form embryoid bodies (EBs). After 8 days, the iPS cells formed round embryoid bodies and we then transferred the EBs to gelatin-coated plates and cultivated them for another 8 days. Immunocytochemical analysis showed that for all the iPS cell lines attached cells derived from the EBs were positive for alpha-fetoprotein (endoderm), alpha-smooth muscle actin (mesoderm), glial fibrillary acidic protein (ectoderm), and beta-3
tubulin (ectoderm) (Figure 2-C-f, g, h, i). To evaluate pluripotency in vivo, we transplanted the human iPS cells into the testes of SCID mice. All of the established human iPS cell lines derived after hypoxic treatment developed teratomas and the histological study showed the cells in the teratomas to differentiate into tissues representing all three germ layers (Figure 2-E).

Although hypoxic conditions promote reprogramming, hypoxia also induces cytotoxicity. There are significant differences between cell types in terms of their susceptibility to hypoxia. In our experiments, HDFs were more susceptible to hypoxic cytotoxicity than MEFs. Cultivation under 1% O₂ inhibited the proliferation of HDFs and even led cell death within several days, while cultivation under 1% O₂ had little effect on the proliferation of MEFs. In our experiments, hypoxic cultivation showed no significant effects on the survival of mouse ES cells and four-factor transduced cells, or on the proliferation of mouse ES cells and mock transduced MEFs. However, in four-factor transduced MEFs hypoxia showed a significant proliferative effect and increased the expression level of Oct3/4 and Nanog. In addition, exposure of MEFs to hypoxic conditions shifted the overall gene expression pattern towards that of ES cells. Although there may be several explanations for the positive effect of hypoxia on the efficiency of reprogramming, these results suggest that hypoxic conditions may contribute to the reprogramming process itself.
In this study, we created hypoxic conditions by flushing hypoxic gas mixture, using Forma Series II Universal CO$_2$ incubators (Thermo scientific, Massachusetts), in which mild hypoxia (5-6% O$_2$) in a gas phase can be achieved within 10 minutes after opening and closing of the door. However, since we changed the medium in a laminar flow hood under normoxic atmosphere, there must have been some fluctuation in O$_2$ content after medium change. More strict control of hypoxia, using a closed hypoxia workstation or medium pre-equilibrated under hypoxic conditions, might further increase the efficiency of iPS cell generation.

In summary, by comparing the efficiency of iPS cell induction under normoxic and hypoxic conditions, we showed that hypoxic conditions can improve the efficiency of iPS cell generation from mouse and human somatic cells. We have found that cultivation under 5% O$_2$ favors more efficient iPSC generation, but further characterization is needed to determine the optimal O$_2$ concentration and duration of hypoxic culture for promoting reprogramming process. Ultimately, we hope that understanding the basis of this effect of hypoxia will contribute to ongoing efforts to devise a methods for efficient iPSC that does not require genetic modification.
Figure legends

Figure 1. Hypoxia promotes Reprogramming of Mouse Embryonic Fibroblasts to iPS Cells.

(A) Counts of the Nanog-GFP-positive colonies from four-factor transduced MEFs on day 21 (white) and on day 28 (black) (a), from three-factor transduced MEFs on day 21 (white) and on day 28 (black) (b). The percentage of GFP-positive colonies in total colonies from four-factor transduced MEFs (c) and three-factor transduced MEFs (d) on day 21. (e) Counts of Nanog-GFP-positive colonies from Oct3/4 and Klf4-transduced MEFs on day 28. The averages and standard deviations of four experiments are shown. * p<0.05 vs. 21% O₂, ** p<0.01 vs. 21% O₂.

(B) Percentage of GFP-positive cells from four-factor transduced MEFs on day 9 cultivated under hypoxic and normoxic conditions with and without VPA. Representative flow cytometric analysis of four-factor transduced MEFs under 21% O₂ (a) and 5% (b) without VPA, and under 21% O₂ (c) and under 5% (d) with VPA. The signal from the PE channel was used as a control for autofluorescence. The averages and standard deviations of three independent experiments are shown. * p<0.05 vs. 21% O₂ without VPA, ** p<0.0001 vs. 21% O₂ with VPA. p<0.0001 vs. 5% O₂ without VPA.
(C) The iPS cells derived from two-factor transduced MEFs gave rise to chimeric mice with a cinnamon coat color in contrast to wild type ICR mice. Chimeric mouse from MEF-2F-iPS (a) and wild type ICR mouse (b).

(D) MEFs were transiently transfected with expression plasmids of reprogramming factors, and cultivated under hypoxic and normoxic conditions. On day 25, the number of GFP-positive colonies was counted. Counts of the Nanog-GFP-positive colonies from six experiments were plotted. * p<0.05 vs. 21% O₂

(E) MEFs were reprogrammed using doxycycline-inducible transcription factors delivered by PB transposition. The cells were cultivated under hypoxic or normoxic conditions. Counts of the Nanog-GFP-positive colonies on day 12 were shown. The averages and standard deviations of three experiments are shown. * p<0.01 vs. 21%O₂, ** p<0.001 vs. 21%O₂

Figure 2. Hypoxia Increases the Efficiency of iPS Cell Generation from Human Dermal Fibroblasts.

(A) Time schedules of human iPS cell generation from HDFs.

(B) The number of human ES-like colonies on day 24 (a), on day 32 (b), and on day 40 (c). The averages and standard deviations of three experiments are shown. * p<0.01 vs. 21% O₂. **
p<0.05 vs. 21% O2.

(C) Representative phase contrast image of human ES-like colonies (a) and alkaline phosphatase staining of the established iPS clone generated under 5% O2 (b). Immunohistochemical staining of undifferentiated human iPS cells generated under hypoxic conditions. Nanog (c), SSEA3 (d), SSEA4 (e). Immunohistochemical staining of three germ layer markers in the differentiated human iPS cells generated under hypoxic conditions. Alpha-fetoprotein (f), alpha-smooth muscle actin (g), glial fibrillary acidic protein (h), beta-3 tubulin (i). Scale bars, 100μm.

(D) Semiquantative RT-PCR analysis of ES cell-marker genes in human iPS cells generated under normoxic and hypoxic conditions and human ES cells (hES3).

(E) Teratoma formation of human iPS cells generated under hypoxic conditions. Neural epithelium (a), pigmented retinal epithelium (b), bone-like structure (c), smooth muscle cells (d), endodermal epithelium (e). Scale bars, 100μm.

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References


Supplemental Figure Legends

Figure S1 The size and morphology of GFP-positive colonies cultivated under hypoxic conditions were comparable to those derived under normoxic conditions.

The representative images of GFP-positive colonies under 21% O₂ (A; phase contrast, B; GFP) and 5% O₂ (C; phase contrast, D; GFP) photographed on day 21 were shown. Scale bars, 200μm.

Figure S2 Improved efficiency of alkaline phosphatase-positive colony generation under hypoxic conditions.

Representative images of four-factor infected MEFs on day 21 under 21% O₂ (A) and 5% O₂ (B), three-factor infected MEFs on day 28 under 21% O₂ (C), and 5% O₂ (D).

Figure S3 iPS cells derived under hypoxic conditions had normal karyotypes.

The iPS clones derived after hypoxic treatment (521AH5-1 and 527CH5-1) showed normal karyotype. Karyotype analysis of 527CH5-1 is shown.
Figure S4  The mRNA expression patterns of iPSCs derived under hypoxia were comparable to those of ES cells

Semiquantitative RT-PCR analysis of ES cell-marker genes in iPSCs generated under hypoxic conditions, ES cells (RF-8), and representative iPS cells (20D17).

Figure S5 iPSCs derived after hypoxic treatment were capable of differentiating into all three germ layers in teratoma formation assays

Hematoxylin and eosin staining of teratoma sections showed the differentiation of iPS cells in three germ layers. (A) keratinized epithelium, (B) neural tissue, (C) cartilage, (D) smooth muscle cells, (E) Endodermal epithelium. Scale bars, 100μm.

Figure S6 Hypoxia had no protective effect on ES cells and reprogramming MEFs.

(A) ES cells (RF8) were seeded onto the feeder layer of STO cells at the density of 1x10⁵ cells/well and cultivated under normoxia or hypoxia from day 1 to 3. On day 3 the cells were treated with annexin V-FITC and subjected to flow cytometric analysis. Bar graphs represent the percentage of apoptotic cells (annexin V-FITC-positive). (B) Four-factor transduced MEFs were seeded onto STO cells on day 4 posttransduction and cultivated under hypoxic and normoxic
conditions from day 5 to day 9, and the cells were subjected to annexin-V affinity assay. Bar graphs represent the percentage of apoptotic cells. The averages and standard deviations of three experiments are shown.

Figure S7 Hypoxia had no effect on the proliferation of ES cells.

ES cells (RF8) were seeded onto the feeder layer of STO cells at the density of 1x10^5 cells/well and cultivated under normoxia or hypoxia from day 1 to 3. On day 3, the number of the cells was counted. Bar graphs show the cell count of ES cells. The averages and standard deviations of three experiments are shown.

Figure S8 Hypoxia significantly increased the number of four-factor transduced MEFs but not mock transduced MEFs.

Four-factor and mock transduced MEFs were cultivated respectively under hypoxic or normoxic conditions from day 1 to 4 and the number of cells were counted. Bar graphs show the cell count. The averages and standard deviations of four experiments are shown. * p<0.05

Figure S9 Hypoxia enhanced the expression of reprogramming-related genes.
MEFs were cultivated under hypoxic or normoxic conditions for 10 days, and the RNA was extracted and subjected to microarray analysis (A, B). Four-factor transduced MEFs were cultivated under hypoxic or normoxic conditions from day 1 to day 4, and the RNA was extracted and subjected to microarray analysis and quantitative real-time RT-PCR (C, D, E, F). (A, B) Scatter plots of expression patterns of ES cell-specific genes (A) and MEF-specific genes (B) comparing cultivated MEFs under 5% O_2 with those under 21% O_2 and expression patterns of ES cell-specific genes (C) and MEF-specific genes (D) comparing four-factor transduced MEFs under 5% O_2 with those under 21% O_2. Genes that were specifically expressed in ES cells and MEFs were selected (more than tenfold difference). Up- and down-regulated genes in four-factor transduced MEFs with hypoxic treatment are shown in red and blue, respectively. Green lines indicate 5-fold changes in gene expression levels. (E, F) The expressions of endogenous Oct3/4 and Nanog were quantified by real-time RT-PCR. Bar graphs represent the expression of Oct3/4 (E) and Nanog (F). The averages and standard deviations of three experiments are shown. * p<0.001, ** p<0.05

**Figure S10 Hypoxia enhances the reprogramming of MEFs without STO feeder cells.**

Four-factor transduced MEFs were seeded onto a gelatin-coated dish at the density of 2x10^5
/dish and cultivated under hypoxic or normoxic conditions. (A) Counts of the Nanog-GFP-positive colonies on day 21. The representative image of GFP-positive colonies derived under 21% O$_2$ (B; phase contrast, C; GFP) and under 5% O$_2$ (D; phase contrast, E; GFP). The averages and standard deviations of three experiments are shown. Scale bars, 200μm. * p<0.05

Supplemental Methods

The generation of mouse and human induced pluripotent stem cells (iPS cells) was performed as previously described (Okita et al., 2007; Takahashi et al., 2007; Takahashi and Yamanaka, 2006) with some modifications. Mouse embryonic fibroblasts (MEFs), which contained the Nanog-GFP-IRES-Puro' reporter, were seeded at 1.0x10$^5$ cells/ well in 6-well plates and then the next day four, three, or two transcriptional factors were retrovirally introduced. On day 4 posttransduction, the cells were trypsinized, and the four factor-transduced MEFs were seeded at 1x10$^4$ cells/ 100-mm dish, and three or two factor-transduced MEFs were seeded at 1x10$^5$ cells/ 100-mm dish onto the feeder layer of mitomycin C-treated STO cells. The number of GFP-positive colonies was thereafter counted on day 21 and day 28. Human dermal fibroblasts
(HDFs) were seeded at 3.0x10^5 cells/ 60-mm dish and the next day the cells were infected with retrovirus delivering four transcriptional factors. On day 6 posttransduction, the cells were trypsinized and seeded onto the feeder layer of mitomycin C-treated STO cells at 1x10^5 cells/ 100-mm dish. The number of human embryonic stem cell-like colonies was then counted on day 40. Hypoxic conditions were created and sustained by flushing hypoxic gas mixture, using Forma Series II Universal CO2 incubators (Thermo scientific, Massachusetts).

Generation of iPS cells with plasmid transfection was performed as previously described (Okita et al., 2008). Briefly, MEFs, which contain Nanog-GFP-IRES-Puro reporter, were seeded at 1.0x10^5 cells/ well in 6-well plates (Day0). On day 1, 3, 5, and 7, the cells were transfected with pCX-OKS-2A and pCX-c-Myc, and on day 9, the cells were harvested with trypsin and were reseeded onto 100-mm dishes with STO feeder cells. On day 25, the number of GFP-positive colonies was counted. For hypoxic treatment, the cells were cultivated under 5% O2, from day 10 to day 24.

Direct reprogramming with piggyBac (PB) transposition was performed as previously described with some modifications (Woltjen et al., 2009). Briefly, MEFs, which contain Nanog-GFP-IRES-Puro reporter, were seeded at 1.0x10^5 cells/ well in 6-well plates. After 24h culture, Fugene HD (Roche, Switzerland) was used to transfect cells with PB-TET-MKOS,
PB-CA-rtTA Adv, and PB transposase expressing vector. After 24h, the media was replaced with doxycycline-containing media (1.5ug/ml) (Day 0). The cells were cultivated under hypoxic or normoxic conditions, and the number of GFP-positive colonies were counted on day 12. PB-TET-MKOS and PB-CA-rtTA adv were provided from Addgene (Addgene plasmid 20910 and 20959). The PB transposase construct was amplified by PCR from pBSII-IFP2-orf (a generous gift from Dr. Malcolm J. Fraser, Jr, University of Notre Dame), and was inserted into the expression vector driven by CAG-promoter (pCX-EGFP).

For the fluorescence-activated cell sorting analysis, on day 4 posttransduction, the four factor-transduced cells were trypsinized and seeded onto the feeder cells at 2x10³ cells in 6-well plates. On day 9 posttransduction, the cells were trypsinized and then were subjected to flow cytometric analysis. For the apoptosis detection assay, on day 9 posttransduction, the four-factor transduced cells were trypsinized and stained with annexin V-FITC (BioVision, California) and subjected to flow cytometric analysis.

Immunocytochemistry, alkaline-phosphatase staining, and RT-PCR were all performed as previously described (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

References


Figure S2

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Figure S4

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- Oct3/4 (end)
- Oct3/4 (Tg)
- SOX2 (end)
- Klf4 (end)
- c-Myc (end)
- Nanog
- Rex1
- ECAT1
- NAT1
- RT- (NAT1)
Figure S6

A  ES cells

B  Four-factor transduced MEFs

% apoptotic cells

21%  5%

% apoptotic cells

21%  5%
Figure S7

ES cells

Cell count

21% 5%
Figure S8
Figure S10

A

Number of GFP+ colonies

Mock 21% 5% 1%

B 21%

C 21%

D 5%

E 5%