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Heterogeneity of pluripotent marker gene expression in colonies generated in human iPS cell induction culture☆

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Abstract Induction of pluripotent stem cells from human fibroblasts has been achieved by the ectopic expression of two different sets of four genes. However, the mechanism of the pluripotent stem cell induction has not been elucidated. Here we identified a marked heterogeneity in colonies generated by the four-gene (Oct3/4, Sox2, c-Myc, and Klf4) transduction method in human neonatal skin-derived cells. The four-gene transduction gave a higher probability of induction for archetypal pluripotent stem cell marker genes (Nanog, TDGF, and Dnmt3b) than for marker genes that are less specific for pluripotent stem cells (CYP26A1 and TERT) in primary induction culture. This tendency may reflect the molecular mechanism underlying the induction of human skin-derived cells into pluripotent stem cells. Among the colonies induced by the four-gene transduction, small cells with a high nucleus-to–cytoplasm ratio could be established by repeated cloning. Subsequently established cell lines were similar to human embryonic stem cells as well as human induced pluripotent stem (iPS) cells derived from adult tissue in morphology, gene expression, long-term self-renewal ability, and teratoma formation. Genome-wide single-nucleotide polymorphism array analysis of the human iPS cell line indicates that the induction process did not induce DNA mutation.

Introduction

Human pluripotent stem cells can be harvested from human blastocysts or from primordial germ cells in human embryos, the former being embryonic stem cells and the latter embryonic germ cells (Shamblott et al., 1998; Thomson et al., 1998). If pluripotent stem cells could be generated from postnatal tissue of an individual patient, these would greatly facilitate the therapeutic application of stem cells by avoiding the immune rejection by the recipient which is associated with allograft transplants (Hochedlinger and Jaenisch, 2003; Yang et al., 2007). They would also have the potential to be used in patient-specific *in vitro* models for studying disease etiology and the role of genetic variation in response to drugs. Somatic cell nuclear transfer and fusion of somatic cells with ES cells have been used for this purpose,

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but technical and ethical issues limit their application (Cowan et al., 2005; Hochedlinger and Jaenisch, 2006; Tada et al., 2001; Yamanaka, 2007). It was shown that ectopic expression of four transcription factors, Oct3/4 (POU5F1), Sox2, c-Myc, and Klf4, in mouse fibroblasts induces pluripotent stem cells (Blelloch et al., 2007; Maherali, 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Germline-competent pluripotent stem cells generated from mouse fibroblasts by the four-gene transduction method were isolated by using Nanog or Oct3/4 as a selection factor (Maherali, 2007; Okita et al., 2007; Wernig et al., 2007). Recently, it was shown that two different sets of four genes can induce pluripotent stem cells from human fibroblasts (Takahashi et al., 2007; Yu et al., 2007). In addition, it was suggested that c-Myc is not necessary for the induction of human induced pluripotent stem (iPS) cells (Nakagawa et al., 2007). However, the mechanism underlying pluripotent stem cell induction has not been elucidated.

Here we used a method for the transduction of human neonatal skin-derived cells that employs adenovirus carrying the ecotropic receptor mCAT1 followed by Moloney murine leukemia virus (MMLV)-derived vectors carrying the four transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (Koch et al., 2006). Using this method, human skin fibroblasts gave rise to colonies expressing alkaline phosphatase (ALP) and Nanog 17 days after the four-gene transduction. However, ALP/Nanog double-positive cells were heterogeneous and included cells lacking expression of one or more of the TDGF1, Dnmt3b, Zfp42, FoxD3, GDF3, CYP26A1, and TERT genes. The four-gene transduction gave a higher probability of induction for archetypal pluripotent stem cell marker genes (Nanog, TDGF1, and Dnmt3b) than for marker genes that are less specific for pluripotent stem cells (CYP26A1 and TERT). This tendency may reflect the molecular mechanism underlying the induction of human skin-derived cells into pluripotent stem cells. Among the colonies induced by the four-gene transduction, small cells with a high nucleus-to-cytoplasm ratio could be established by repeated cloning. Subsequently established cell lines were similar to human embryonic stem cells in morphology, gene expression, long-term self-renewal ability, and teratoma formation. The four transgenes that were used to induce the pluripotent cells were found to be silenced in the established pluripotent cell clones. We also investigated the DNA mutation ratio associated with the process of pluripotent stem cell induction. Genome-wide single-nucleotide polymorphism array analysis was performed for the human iPS clone 1–8 and its parental cell, and no marked differences were observed. Consistent with these observations, the HLA genotype of the human iPS clone and the parental cells was identical. These results indicate that the induction processes do not introduce DNA mutations.

Results

Analysis of colonies induced by ectopic expression of the four genes in human skin-derived cells

Human neonatal skin-derived cells were infected with an adenovirus vector carrying the murine retrovirus receptor mCAT. Subsequently, the cells were exposed to replication-deficient MMLV-derived vectors carrying human Oct3/4, Sox2, c-Myc, and Klf4 cDNA. The cells were fixed and stained for ALP on day 17 after the four-gene transduction, when the cells had become fully confluent. In total, 163 ALP-positive colonies and 80–90 ALP-negative colonies were observed in four independent experiments. All 163 ALP-positive colonies and 18 ALP-negative colonies were isolated for gene expression analysis using real-time polymerase chain reaction with reverse transcription. Eight genes (Nanog, TDGF1, Dnmt3b, Zfp42, FoxD3, GDF3, CYP26A1, and TERT) that are reported to be expressed in human ES cells were selected as pluripotent stem cell marker genes (Adewumi et al., 2007; Assou et al., 2007). Human neonatal skin fibroblasts did not express any of these eight genes. Gene expression profiles of 163 ALP-positive colonies and 18 ALP-negative colonies were categorized according to the number of genes expressed from the panel of these eight ES cell markers and are shown in Table 1. Four of 163 ALP-positive colonies were positive for all eight markers (Table 1). The efficiency of inducing these octa-positive colonies was 0.0005–0.0015% of the initial parent cell count (Fig. 1A).

Nanog gene expression was observed in 161 of 163 ALP-positive colonies and 16 of 18 ALP-negative colonies (Table 1). The pluripotent stem-cell-like colonies (Figs. 1C–1J) as well as fibroblastic colonies (Figs. 1K–1N) expressed Nanog. On the other hand, expression of CYP26A1 and TERT was observed in only 26 and 24 of 163 ALP-positive colonies respectively (Fig. 1B, Table 1). Approximately 90% of TERT-positive cells expressed more than six of the eight ES cell marker genes. Genes such as Nanog, TDGF1, and Dnmt3b, which are well known to be closely associated with the pluripotent state in human ES cells and to be strongly down regulated upon their differentiation, had a high probability of induction by the four-gene transduction. These archetypal pluripotent stem cell marker genes (Nanog, TDGF, and Dnmt3b) were expressed not only in stem-cell-like colonies but also in fibroblastic colonies (Figs. 1K–1N).

ALP-positive colonies could be categorized into 40 groups based on the gene expression pattern of the eight marker genes (Table 1). When colonies were categorized by the total number of the eight marker genes expressed, a binomial distribution was obtained, suggesting that there is a stochastic process in colony induction in addition to the gene selective mechanism (Fig. 1A).

Although the four transgenes were clearly silenced in the iPS clones 94 days after the four-gene transduction (Supplementary Fig. 1K), quantitative gene expression analysis of colonies formed 17 days after infection indicated that the transgenes c-Myc and Oct3/4 showed high expression in all the analyzed colonies (Supplementary Table 1). In addition, endogenous Nanog expression was very high in most of the ALP-positive colonies, including cells lacking expression of one or more of the eight human ES cell marker genes (Supplementary Table 1). These results indicate that the process of pluripotent stem cell induction from human skin-derived cells is slower than that described for mouse iPS cell generation (Maherali, 2007; Okita et al., 2007; Wernig et al., 2007).

Isolation and establishment of human induced pluripotent stem cell lines

Cells of the four octa-positive (Nanog, TDGF1, Dnmt3b, Zfp42, FoxD3, GDF3, CYP26A1, and TERT) colonies showed common

Table 1 Gene expression profiles of ALP(+) and ALP(−) colonies

No.	No. of genes	Nanog	TDGF1	Dnmt3b	Zfp42	FoxD3	GDF3	CYP26A1	TERT	No. of colonies
Gene expression patterns in ALP(+) colonies										
1	8	+	+	+	+	+	+	+	+	4
2	7	+	+	+	+	+	+	+	−	7
3	7	+	+	+	+	+	+	−	+	11
4	7	+	+	+	+	+	−	+	+	1
5	6	+	+	+	+	+	+	−	−	25
6	6	+	+	+	+	+	−	+	−	4
7	6	+	+	+	+	+	−	−	+	3
8	6	+	+	+	+	−	+	−	+	2
9	6	+	+	+	+	−	+	+	−	3
10	6	+	+	+	−	+	+	+	−	1
11	6	+	+	+	−	−	+	+	+	1
12	5	+	+	+	+	+	−	−	−	22
13	5	+	+	+	+	−	+	−	−	9
14	5	+	+	+	+	−	−	+	−	2
15	5	+	+	+	−	+	+	−	−	4
16	5	+	+	+	−	+	−	+	−	2
17	5	+	+	+	−	−	+	+	−	1
18	5	+	+	−	+	+	+	−	−	2
19	5	+	+	−	+	+	−	−	+	1
20	4	+	+	+	+	−	−	−	−	9
21	4	+	+	+	−	+	−	−	−	3
22	4	+	+	+	−	−	+	−	−	5
23	4	+	+	−	+	+	−	−	−	7
24	4	+	−	+	+	+	−	−	−	1
25	4	+	−	+	−	+	+	−	−	2
26	4	+	−	−	+	+	+	−	−	1
27	3	+	+	+	−	−	−	−	−	1
28	3	+	+	−	+	−	−	−	−	3
29	3	+	+	−	−	+	−	−	−	4
30	3	+	+	−	−	−	−	−	+	1
31	3	+	−	+	+	−	−	−	−	1
32	3	+	−	+	−	+	−	−	−	2
33	3	+	−	+	−	−	+	−	−	1
34	3	+	−	−	+	+	−	−	−	1
35	3	+	−	−	−	+	+	−	−	1
36	2	+	+	−	−	−	−	−	−	4
37	2	+	−	+	−	−	−	−	−	5
38	2	+	−	−	+	−	−	−	−	2
39	1	+	−	−	−	−	−	−	−	2
40	0	−	−	−	−	−	−	−	−	2
Gene expression patterns in ALP(−) colonies										
41	6	+	+	+	+	+	−	+	−	1
42	6	+	+	−	+	+	+	−	+	1
43	5	+	+	+	+	+	−	−	−	3
44	5	+	+	−	+	+	−	−	+	6
45	4	+	+	+	−	+	−	−	−	1
46	4	+	+	−	+	+	−	−	−	1
47	4	+	+	+	−	−	−	−	+	1
48	2	+	−	−	−	−	−	−	+	1
49	1	+	−	−	−	−	−	−	−	1
50	1	−	+	−	−	−	−	−	−	1
51	0	−	−	−	−	−	−	−	−	1

features that are consistent with the features of human ES cells: (1) small size with a high nucleus-to-cytoplasm ratio and (2) formation of small monolayer colonies within the space between fibroblasts (Figs. 1C–1F and 2B). Based on these common features, colonies were isolated without ALP staining at 17 to 33 days after four-gene transduction (Fig. 2B).

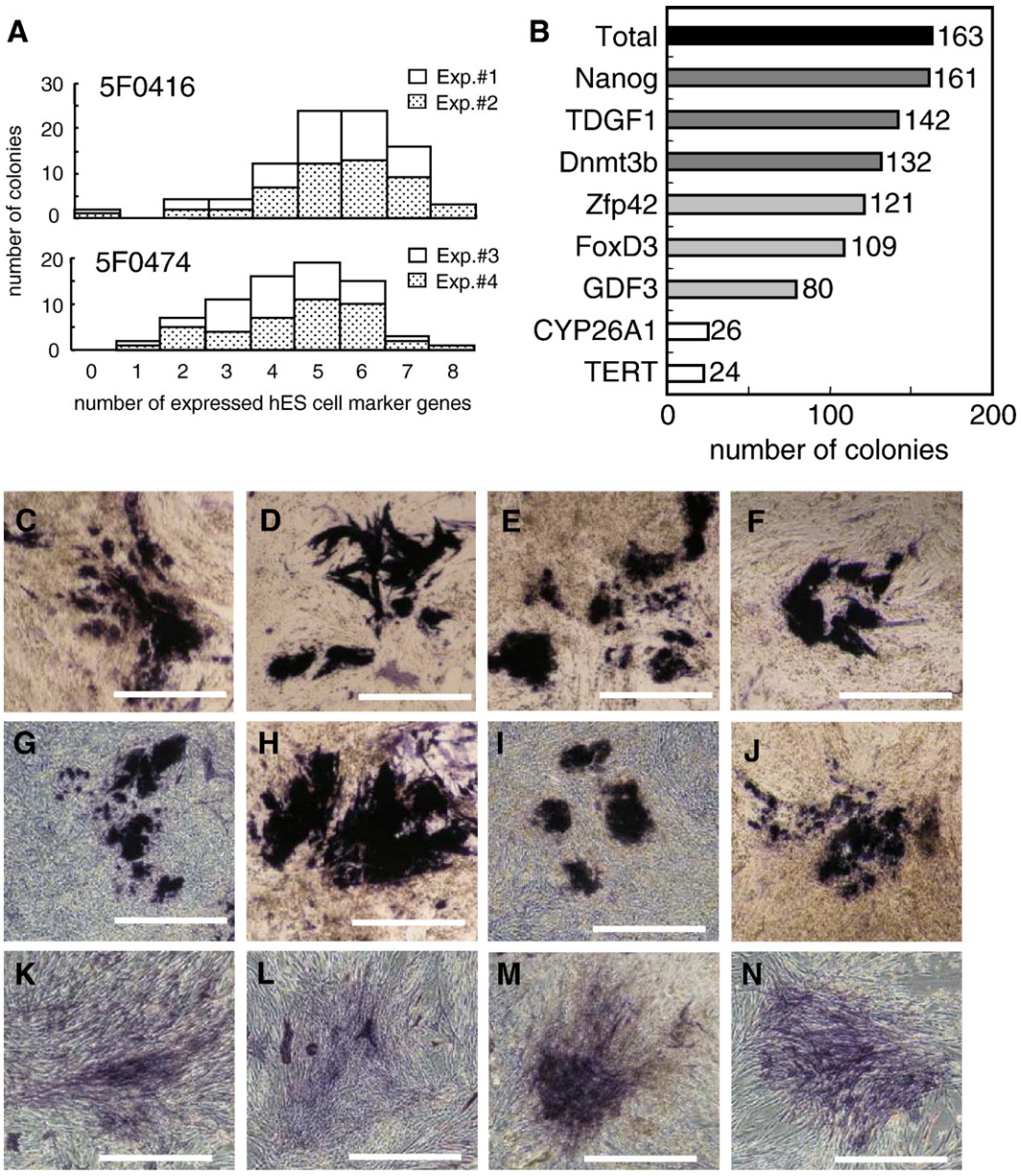

Figure 1 Human ES cell marker gene expression profile in ALP-positive colonies. Colonies were stained for alkaline phosphatase 17 days after four-gene transfer. All ALP$^+$colonies were dissected and their human ES cell marker gene expression was determined. (A) The graph shows the distribution of colonies induced in four independent experiments based on the number of human ES cell marker genes expressed. 2×10^4 cells were infected in Experiments 1 and 3, and 2×10^5 cells were infected in Experiments 2 and 4. (B) The number of colonies expressing Nanog, TDGF1, Dnmt3b, Zfp42, FoxD3, GDF3, CYP26A1, and TERT genes. (C–N) Morphology of human ES-cell-like colonies that expressed all eight of the ES cell marker genes (C–F) or that lack two (G, H) or three (I, J) of the eight marker genes are shown after fixation and staining for ALP. The morphology of fibroblastic colonies that did not express TERT and GDF3 (K, L) or TERT, CYP26A1, and GDF3 (M, N) is also shown. Scale bar, 500 μm.

Approximately half of the colonies isolated by morphological criteria stopped proliferating or changed their morphology during subcloning. Indeed our fixed-colony analysis indicated that some of the ES cell-like colonies that were revealed by the high-density ALP staining in Figs. 1G–1J lack one or more of the eight marker genes.

Three human ES-like cell lines, which we termed human iPS clones 1–8, 2–4, and 3–2, were established by repeated cloning. They were morphologically indistinguishable from typical human ES cells, consisting of small, round cells with a high nucleus-to-cytoplasm ratio and defined edges (Figs. 2C–F). All established lines expressed the eight ES cell marker genes used for the analyses of primary induction cultures (Supplementary Table 2). Efficiency of establishing the human iPS cell lines was about 0.001–0.01% of the initial parent cells, which is consistent with the efficiency of octa-positive colony formation. All three clones could be cultured on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layers as well as under feeder-free conditions in mTeSR1 defined medium. Treatment with the Rho-associated protein kinase inhibitor

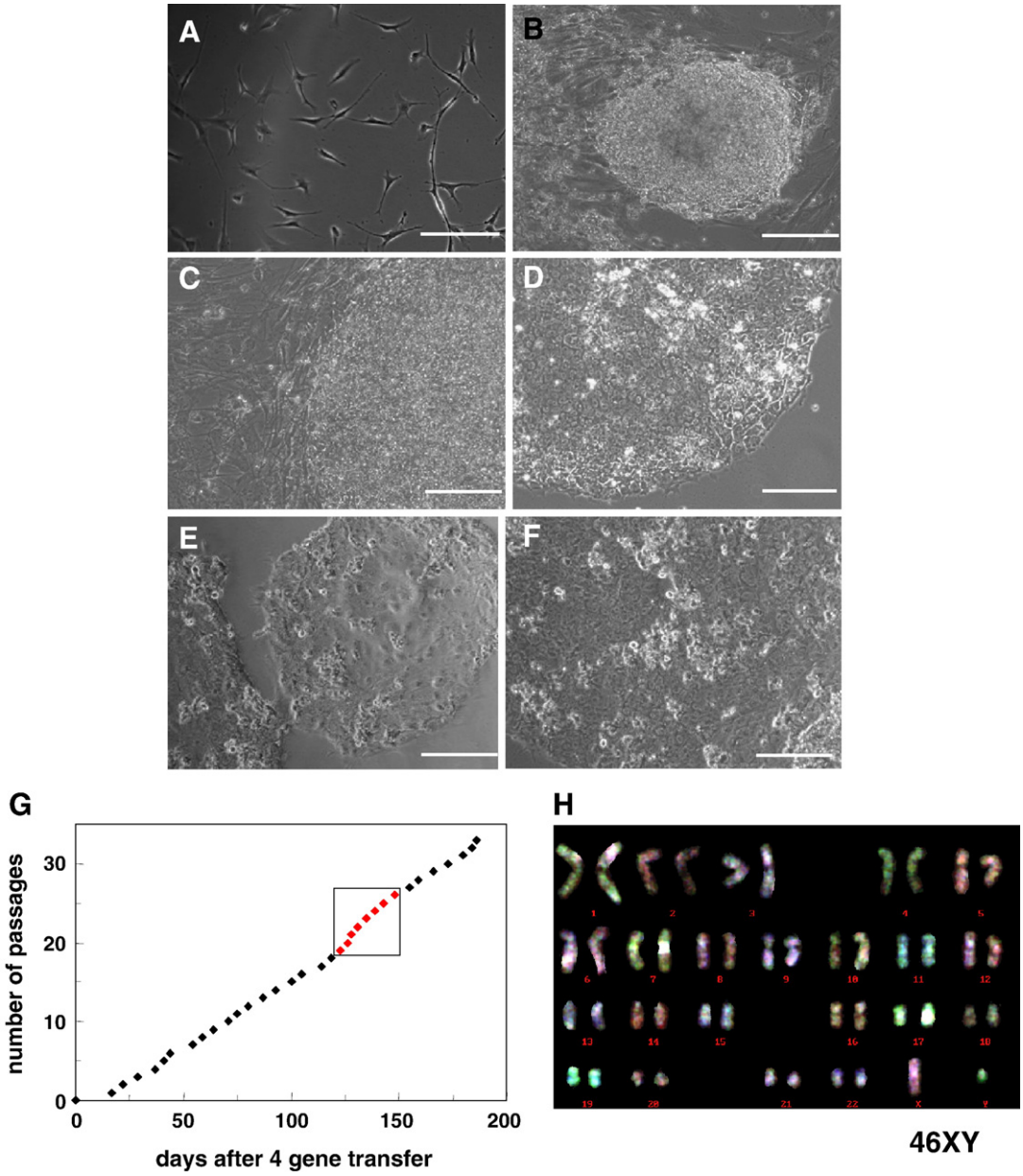

Figure 2 Induction and expansion of pluripotent stem cells. (A, B) Morphology of the parental cells (A) and a colony in primary induction culture, which was later established as human iPS clone 3–2 (B). (C–F) Representative morphology of clone 1–8 cultured on MEF feeder cells (C), and clones 1–8, 2–4, and 3–2 cultured in mTeSR1 medium (D–F). Scale bar indicates 200 μm. (G) Growth record of clone 1–8. The square indicates the period over which cell numbers were counted to estimate cell proliferation rate. (H) Multicolor karyogram image. Karyotype was normal at day 101.

Y-27632 (5 to 20 μM) during passage improved the recovery ratio (Watanabe et al., 2007). Human iPS clone1-8 has been expanded over more than 33 passages, which corresponds to 190 days in culture after the four-gene infection (Fig. 2G).

The population doubling time of human iPS clone 1–8 in feeder-free mTeSR culture medium was approximately 48.5 h when analyzed between passages 19 and 26 (Fig. 2G). Multicolor FISH analysis showed no chromosomal translocation or deletion (Fig. 2H). These results indicate that the induction procedure does not generate chromosomal abnormalities. The human iPS clone 1–8 was positive for ALP as well as the carbohydrate antigens SSEA-3 and SSEA-4, the keratin sulfate antigens TRA-1–60 and TRA-1–81, and the protein antigens CD9, CD24, and Thy1 (Supplementary Figs. 1A–I). They also expressed the human ES cell marker genes Nanog, Oct3/4, Sox2, TDGF1, Dnmt3b, GABRB3, GDF3, TERT, Sall4, Zfp42, and CYP26A1 (Supplementary Figs. 1J and K). The four exogenous transcription factor genes, were silenced in cells from human iPS clone 1–8 at day 94 (Supplementary Fig. 1K).

Global gene expression in human iPS cell lines

The global gene expression profiles of the human iPS lines (1–8, 2–4, and 3–2) and their parental fibroblasts were analyzed

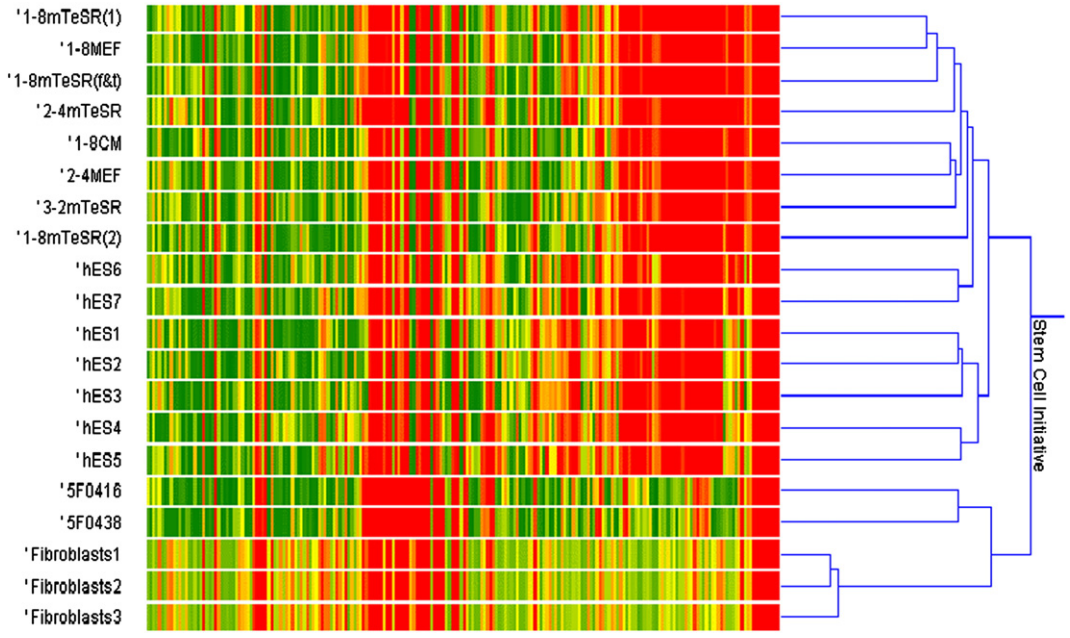

Figure 3 Hierarchical cluster analysis. Two-way cluster analysis was performed for the genes defined by the International Stem Cell Initiative (except for PTF1A). Human iPS clone 1–8 cultured in mTeSR (1–8mTeSR), clone 1–8 cultured in mTeSR after freeze–thaw treatment (1–8 mTeSR(f&t)), clone 1–8 cultured in MEF-conditioned medium (1–8CM), clone 1–8 cultured on MEF (1–8MEF), clone 2–4 cultured in mTeSR (2–4mTeSR), clone 2–4 cultured on MEF (2–4MEF), clone 3–2 cultured in mTeSR (3–2mTeSR), their parental cells (5F0438 and 5F0416), human ES cell line Sheff 4 cultured on MEF (hES1, hES2, hES3), human ES cell line Sheff 4 cultured on Matrigel (hES4, hES5), human ES cell line H14 cultured on MEF (hES6, hES7), and fibroblast data from the GEO database (Fibroblasts1, 2, and 3) were used for the analysis. Expression intensity ranges from red (high) to green (low).

using microarray technology. Hierarchical cluster analysis using the gene set defined by the International Stem Cell Initiative (Supplementary Table 6) (Adewumi et al., 2007) revealed that the human iPS lines (1–8, 2–4, and 3–2) clustered with human ES cell lines but separated from their parental skin-derived cells (Fig. 3). This analysis indicates that the difference between the human iPS cell lines and an established human ES cell line (H14) are smaller than the differences among established ES cell lines. Representative scatter plots indicated that the human ES cell marker genes, Nanog, Oct3/4, TDGF1, Dnmt3b, GABRB3, GDF3, Zfp42, and ALP, showed a high correlation between human iPS cell line 1–8 and human ES cell line H14 (Supplementary Fig. 2A). In contrast, these genes did not correlate between the human iPS cell line 1–8 and its parental cells (Supplementary Fig. 2B). The human iPS cell lines (1–8, 2–4, and 3–2) established from neonatal skin-derived cells in this study were similar in terms of global gene expression to a human iPS cell line (201B) established from adult fibroblasts (Table 2).

DNA methylation of human iPS cell lines

To investigate the DNA methylation status of Nanog and Oct3/4 promoter regions of human iPS cell lines 1–8 and 2–4, bisulfite genomic DNA fragments were analyzed using MALDI-TOF MS. The Oct3/4 proximal promoter including conserved region 1 (CR1), the Oct3/4 promoter distal enhancer including CR4, and the Nanog proximal promoter including Oct3/4 and Sox2 binding sites were examined (Fig. 4A) (Rodda et al., 2005; Yang et al., 2005). As shown in Fig. 4B, cytosine–phosphate–guanosine (CpG) dinucleotides in these regions were demethylated in human iPS cell lines 1–8 and 2–4 compared to their parental cells.

Pluripotency of the human iPS cell line

Human iPS clone 1–8 gave rise to teratomas 4 to 8 weeks after transplantation into the testis of SCID mice. The teratomas contained tissues representative of all three germ layers,

Table 2 Pearson correlation coefficients between human iPS cell lines from adult or neonate and human ES cell lines

	hiPS 1–8mTeSR	hiPS 2–4mTeSR	hiPS 3–2mTeSR	hiPS 201B	hES H9	hES ES01	hES BG03
hiPS 2–4mTeSR (neonate)	0.959						
hiPS 3–2mTeSR (neonate)	0.935	0.919					
hiPS 201B (adult)	0.868	0.845	0.908				
hES H9 (embryo)	0.915	0.896	0.889	0.902			
hES ES01(embryo)	0.923	0.902	0.897	0.906	0.982		
hES BG03 (embryo)	0.939	0.923	0.913	0.885	0.974	0.975	
Fibroblasts (adult)	0.516	0.515	0.561	0.621	0.525	0.530	0.509

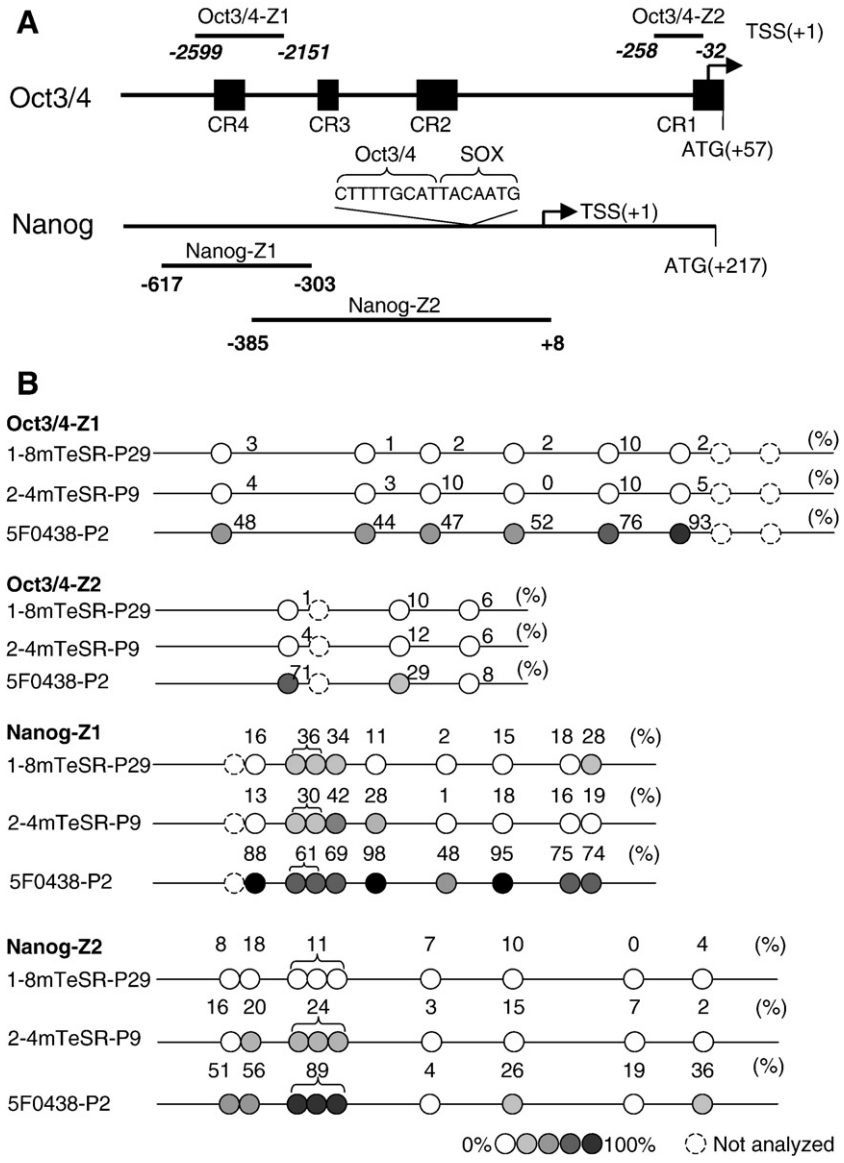

Figure 4 Methylation pattern of Nanog and Oct3/4 promoter regions. (A) Parts of the Oct3/4 promoter including the distal enhancer (Oct3/4-Z1) and the proximal promoter region (Oct3/4-Z2) and parts of the Nanog promoter including the proximal promoter region (Nanog-Z1,-Z2) were analyzed for methylation of CpG. (B) All of the CpG sites in the promoter region defined in (A) are indicated by the circles from 5′ (left) to 3′; (right).

neuroectoderm, mesoderm, and endoderm. Immunohistological analyses revealed that neural epithelium, cartilage, smooth muscle, and endodermal tract were formed as multiple tissues from each germ layer, demonstrating the pluripotency of human iPS clone 1–8 (Fig. 5, Supplementary Fig. 3). Pluripotency was maintained after freeze–thaw treatment of human iPS clone 1–8 (Supplementary Figs. 3T–F1 and 3T–F2).

Genotyping of the human iPS cell lines

Southern blot and genomic-PCR analysis indicated that human iPS clone 1–8 carries ectopically transduced Oct3/4, Sox2, c-Myc, and Klf4 genes in the genome (Supplementary Fig. 4), suggesting that the two-step infection procedure was effective. The other two clones also carried the four transgenes (data not shown).

To investigate the DNA mutation ratio associated with the process of pluripotent stem cell induction, genome-wide single-nucleotide polymorphism array analysis was performed for human iPS clone 1–8 ($n=2$), its parental skin-derived cells ($n=2$), and skin cells derived from another donor ($n=1$). No marked differences were observed between human iPS clone 1–8 and the parental cells (Table 3). Consistent with these observations, HLA genotypes of human iPS cell lines 1–8, 2–4, and 3–2 were identical to those of their respective parental cells (Supplementary Table 3).

Discussion

Our results demonstrate that introduction of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, is sufficient to induce

human ES-cell-like pluripotent stem cells from cells obtained from neonatal human skin tissues, indicating that neonatal-derived tissues as well as adult-derived tissues have the competence to generate iPS cells. The induced pluripotent stem cells were similar to human embryonic stem cells by the criteria of morphology, gene expression, DNA methylation, teratoma formation, and long-term self-renewal ability (Adewumi et al., 2007; Thomson et al., 1998). Chromosomal structures and genome sequences were maintained during the induction process, indicating that pluripotent stem cells generated by this method may have tremendous potential for therapeutic applications as well as for patient-specific *in vitro* models to investigate disease etiology and the role of genetic variation in response to drugs.

Strong silencing of the four ectopically expressed genes indicates that these four genes are not necessary for the maintenance of induced pluripotent stem cells. This is similar to the situation described for mouse Nanog-iPS cells (Maherali, 2007; Okita et al., 2007; Wernig et al., 2007). The difference in the kinetics of pluripotent stem cell induction after the four-gene transduction between mouse and human cells could be caused by the slower doubling time and the slower silencing kinetics of the ectopically expressed genes in human cells (Koch et al., 2006). The ratios of ES-cell-like colonies to Nanog-positive colonies in the primary induction culture were similar for the mouse and human systems (Wernig et al., 2007). The efficiency of establishing human induced pluripotent stem cells was more than 1 order of magnitude lower than that for mouse induced pluripotent stem cells in our experiments.

Table 3 Consistent SNP (%) between human iPS clone1–8, its parental skin-derived cells (5F0438), and skin cells derived from a different donor (5F0416)

	iPS 1–8_01	iPS 1–8_02	5F0438_01	5F0438_02
iPS 1–8_02	99.41			
5F0438_01	99.17	99.26		
5F0438_02	99.44	99.44	99.32	
5F0416	60.50	60.75	60.72	60.47

Although Nanog gene expression is an important marker for the induction of mouse iPS cells (Okita et al., 2007; Wernig et al., 2007), we identified a marked heterogeneity in Nanog-positive colonies generated by the four-gene transduction method in human neonatal skin-derived cells. Archetypal pluripotent stem cell marker genes such as Nanog, TDGF1, and Dnmt3b showed a higher probability of being induced by the four-gene transduction compared to marker genes that are less specific for pluripotent stem cells (CYP26A1 and TERT) (Adewumi et al., 2007; Assou et al., 2007). This tendency might suggests that factors that activate gene expression of marker genes that are less specific for pluripotent stem cells may stimulate the induction of human iPS cells. On the other hand, it is well known that skin-derived cells contain multiple somatic stem cells, progenitor cells, and somatic cells (Blanpain and Fuchs, 2006; Sorrell and Caplan, 2004). Thus heterogeneity of cells in the tissue may contribute to the heterogeneity of colonies in primary induction culture and may

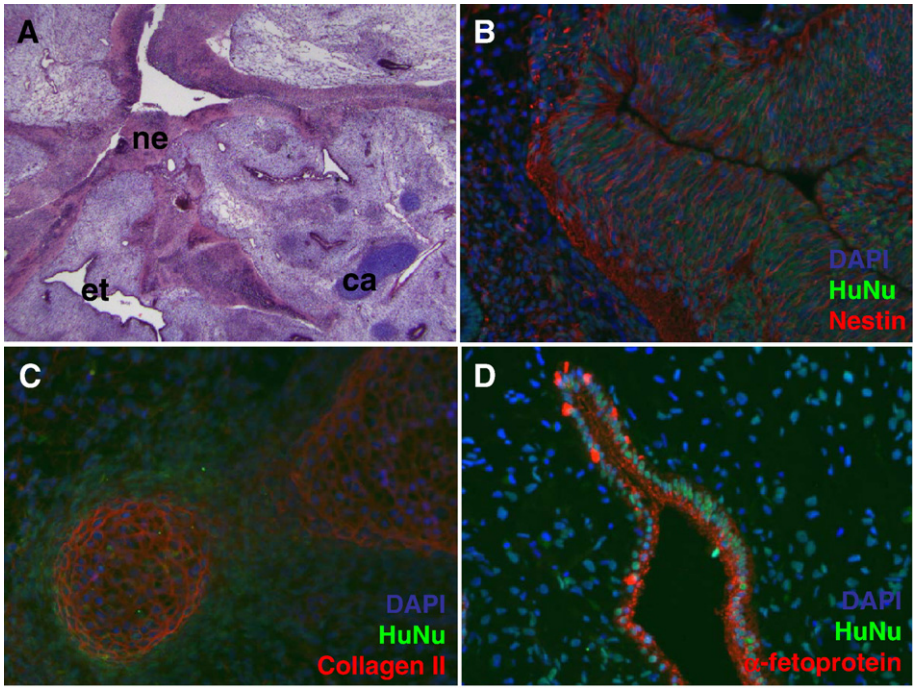

Figure 5 Pluripotency of the human iPS cell clone 1–8. Cells of human iPS clone 1–8 were injected into SCID mouse testis and analyzed 56 days after injection. (A) HE and Alcian blue staining of formaldehyde-fixed teratoma sections. The teratomas contained tissues representative of the three germ layers. Ne, neural epithelium; ca, cartilage; et, endodermal tract. (B–D) Tissues originating from the transplant were distinguished from host tissues by HuNu staining. Nestin-expressing neural epithelium (B), collagen II-expressing chondrocytes (C), and α-fetoprotein-expressing endodermal tract (D) are shown.

influence the efficiency of pluripotent stem cell induction. In addition, culture conditions during *in vitro* propagation of the cell sources of human iPS cells may contribute to the heterogeneity of colonies in the primary induction culture by altering the epigenetic state of the parent cells.

If epigenetic reprogramming is involved in the process of human iPS cell induction, DNA demethylation should play an important role in this process. Although the mechanism of DNA demethylation remains unclear and controversial, four classes of mechanism have been proposed: removal of the methyl moiety from the base, excision of the methylated base, excision of the methylated nucleotide, or cleavage of the DNA backbone at the 3′ site of methylcytidine followed by the DNA repair process (Kress et al., 2001, 2006). In addition, failure to methylate following DNA replication may be a possible mechanism underlying promoter activation of the pluripotency genes. Our genome-wide SNP analysis indicated no marked differences between human iPS cells and the parental cells. In addition, it is important to consider the possibility that rare naïve adult progenitor cells present in the somatic tissue with a similar DNA methylation state to iPS cells serve as the source of iPS cells. Further studies are necessary to identify the molecular mechanisms underlying induction of pluripotency in the parental cells.

Methods

Viral infection and induction of human neonatal skin-derived cells

Adenovirus vector plasmids for mCAT1 were transfected into HEK293 cells. The mCAT1 adenoviruses were isolated from these cells by three freeze–thaw cycles, purified using an adenovirus purification kit (Clontech), and stored at −80 °C. The titer of the vector stocks was determined using an Adeno-X rapid titer kit (Clontech).

The replication-deficient MMLV-derived retrovirus vector pMX (Kitamura et al., 2003) was used for the ectopic expression of human Oct3/4, Sox2, c-Myc, and Klf4. Recombinant retroviruses were generated by transfecting vectors to the Plat-E packaging system (Morita et al., 2000) followed by incubation in FBM (Lonza) supplemented with FGM-2 SingleQuots (Lonza). Between 24 and 48 h after the transfection, supernatant from the Plat-E culture was collected several times at intervals of at least 4 h and passed through a 0.45-μm filter.

For MEF-conditioned medium (MEF-CM) preparation, human ES medium (DMEM/F12; Gibco) supplemented with 20% Knockout Serum Replacement (Invitrogen), 2 mM L-glutamine (Sigma), 1 × nonessential amino acids (Sigma), 10 μg/ml gentamycin (Gibco), and 10 ng/ml bFGF (Peprotech) was conditioned on mitomycin C-treated MEFs (Reprocell) for 20–24 h, harvested, filtered through a 0.45-μm filter, and supplemented with 0.1 mM 2-mercaptoethanol (Sigma) and 10 ng/ml bFGF before use.

Fibroblasts were obtained from human neonatal foreskin, by biopsy under informed consent (Lonza, followed by culture in FBM supplemented with FGM-2 SingleQuots. Cells derived from five different donors were used in this study (5F0416, 5F0438, 5F0439, 5F0474, 5F1195). Three days before the four-gene introduction, cells were seeded at 10^3–10^4 cells/cm^2 into six-well plates. Ten to 18 h later, the cells were mixed with the mCAT1 adenovirus vector solution in 500 μl Hanks' balanced salt solution and incubated at room temperature for 30 min. The cells were then added to 2 ml of medium and cultured for 48 h. Subsequently, the cells were incubated in 2 ml of the retrovirus/Polybrene solution (mixture of equal volumes of the retrovirus vector suspension for each of the four genes, supplemented with 5 μg/ml Polybrene) at 37 °C for 4 h to overnight. The medium was changed from retrovirus/Polybrene solution to MEF-CM immediately after the infection. Medium was changed every 1–2 days.

Cell maintenance of human induced pluripotent stem cell lines

Clone isolation experiments and colony analyses were performed as separate experiments. Colony analyses (shown in Fig. 1) were performed using two different donor cell lots: 5F0416 and 5F0474. Colonies induced by the ectopic expression of four genes were isolated on day 17 to 33 after the four-gene transduction by using a cloning cylinder or forceps. Human iPS clones 1–8 and 3–2 were established from human neonatal dermal cells of Lot 5F0438 and human iPS clone 2–4 was established from human neonatal dermal cells of Lot 5F0416. Human induced pluripotent stem cell lines were established by repeated cloning on a feeder layer of MEFs in human ES medium supplemented with 0.1 mM 2-mercaptoethanol and 10 ng/ml bFGF or on Matrigel (BD; #354230 or #354277) -coated plates in mTeSR1 medium (Stem Cell Technologies).

For passaging, cells were usually treated with the ROCK inhibitor Y-27632 (Calbiochem) as previously described, to prevent apoptosis. Cells were washed with Hanks' balanced salt solution, incubated in 0.25% trypsin–EDTA (Gibco) at 37 °C for 3 min, and then added to the culture medium. Cells were centrifuged at 300*g* at 4 °C and the supernatant was removed. The cells were resuspended in culture medium with 5 to 20 μM Y-27632. The passages were split at 1:4 to 1:6.

Human induced pluripotent stem cell lines were frozen using Cell Freezing Solution for ES cells (Reprocell) according to the manufacturer's manual.

Alkaline phosphatase staining

Cells were fixed with 10% neutral-buffered formalin solution (Wako) at room temperature for 5 min, washed with PBS, and incubated with alkaline phosphatase substrate (One-Step NBT/BCIP; Pierce) at room temperature for 20–30 min. Cells positive for alkaline phosphatase activity were stained blue-violet.

Real-time PCR gene expression analyses

For the quantitative analysis of gene expression in colonies generated in primary induction culture, total RNA was extracted from fixed colonies using the RecoverAll Total Nucleic Acid Isolation kit (Ambion). After cDNA preparation, genes of interest were amplified using TaqMan preamp (Applied Biosystems). Real-time quantitative PCR was performed with an ABI Prism 7900HT (Applied Biosystems) using the following PCR primer sets (Applied Biosystems) Nanog,

Hs02387400_g1; TERT, Hs00162669_m1; GDF3, Hs00220998_m1; CYP26A1, Hs00175627_m1; GAPDH, Hs99999905_m1; Dnmt3b, Hs00171876_ml; FoxD3, Hs00255287_s1; Zfp42, Hs01938187_s1; and TDGF1, Hs02339499_g1. Standard curves were generated for each primer pair. All expression values were normalized to GAPDH.

Teratoma formation

iPS cell suspension (0.5 to 2×10^6 cells/mouse) was injected into the medulla of the left testis of 7-to 8-week-old SCID mice (CB17; Oriental Yeast) using a Hamilton syringe. After 6 to 8 weeks, the teratomas were excised after perfusion with PBS followed by 10% buffered formalin and subjected to histological analysis.

Histology and immunohistochemistry

Teratomas were embedded in mounting medium, and 10 μm frozen sections were prepared. Serial sections were stained with hematoxylin–eosin (HE) to visualize the general morphology. For the detection of cartilage, Alcian blue staining was employed alone or in combination with HE.

For immunostaining, sections were treated with Immunoblock (Dainippon–Sumitomo) for 30 min to block nonspecific binding. Slides were incubated with the following primary antibodies: anti-nestin polyclonal antibody (PRB-570C, Covance, 1:300), anti-type II collagen polyclonal antibody (LB-1297, LSL, 1:200), anti-smooth muscle actin polyclonal antibody (RB-9010-R7, Lab Vision, 1:1), anti-α-fetoprotein polyclonal antibody (A0008, Dako, 1:500), anti-MUC-1 polyclonal antibody (RB-9222-P0, Lab Vision, 1:100), and anti-human nuclei monoclonal antibody (HuNu) (MAB1281, Chemicon, 1:300). For type II collagen, the sections were incubated with hyaluronidase (25 mg/ml) for 30 min before the treatment with primary antibodies. Localization of antigens was visualized by using appropriate secondary antibodies (Alexa Fluor 594 and 688, Molecular Probes, 1:600). Nuclei were stained with DAPI (InnoGenex). Immunostained teratoma sections were analyzed by fluorescence microscopy (Axio Imager Z1, Zeiss).

Karyotype analysis

Karyotype analysis of long-term cultured human iPS cells was performed using multicolor FISH analysis. Human iPS clone 1–8-derived cells were pretreated with 0.02 μg/ml colecemid (Nacalai) for 2–3 h, incubated with 0.06–0.075 M KCl for 20 min, and then fixed with Carnoy's fixative. For multicolor FISH analysis, cells were hybridized with the multicolor FISH probe (Cambio) and analyzed using a DMRA2 fluorescence microscope (Leica).

DNA microarray analysis

The microarray study was carried out using Human Genome U133 Plus 2.0 gene expression arrays (Affymetrix) and Whole Human Genome Oligo microarrays (Agilent). Detailed methods are available in the Supplementary Information.

Methylation analysis

The promoter regions of Nanog and Oct3/4 were analyzed for methylation of individual CpG sites. Ten nanograms of bisulfite-treated genomic DNA was PCR-amplified with primers containing a T7 promoter (Supplementary Table 5), and transcripts were treated with RNase A. Methylation of individual CpG sites was assessed using a MALDI-TOF mass spectrometry-based method (Ehrich et al., 2005) (Epityper, Sequenom). Mass spectra were collected using a MassARRAY mass spectrometer (Bruker Sequenom). Spectra were analyzed using proprietary peak picking and signal-to-noise calculations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2008.01.001.

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