ARTICLES

Generation of germline-competent induced pluripotent stem cells

Keisuke Okita[1], Tomoko Ichisaka[1,2] & Shinya Yamanaka[1,2]

We have previously shown that pluripotent stem cells can be induced from mouse fibroblasts by retroviral introduction of Oct3/4 (also called Pou5f1), Sox2, c-Myc and Klf4, and subsequent selection for *Fbx15* (also called *Fbxo15*) expression. These induced pluripotent stem (iPS) cells (hereafter called Fbx15 iPS cells) are similar to embryonic stem (ES) cells in morphology, proliferation and teratoma formation; however, they are different with regards to gene expression and DNA methylation patterns, and fail to produce adult chimaeras. Here we show that selection for *Nanog* expression results in germline-competent iPS cells with increased ES-cell-like gene expression and DNA methylation patterns compared with Fbx15 iPS cells. The four transgenes (*Oct3/4*, *Sox2*, *c-myc* and *Klf4*) were strongly silenced in Nanog iPS cells. We obtained adult chimaeras from seven Nanog iPS cell clones, with one clone being transmitted through the germ line to the next generation. Approximately 20% of the offspring developed tumours attributable to reactivation of the *c-myc* transgene. Thus, iPS cells competent for germline chimaeras can be obtained from fibroblasts, but retroviral introduction of c-Myc should be avoided for clinical application.

Although ES cells are promising donor sources in cell transplantation therapies[1], they face immune rejection after transplantation and there are ethical issues regarding the usage of human embryos. These concerns may be overcome if pluripotent stem cells can be directly derived from patients' somatic cells[2]. We have previously shown that iPS cells can be generated from mouse fibroblasts by retrovirus-mediated introduction of four transcription factors (Oct3/4 (refs 3, 4), Sox2 (ref. 5), c-Myc (ref. 6) and Klf4 (ref. 7)) and by selection for *Fbx15* expression[8]. Fbx15 iPS cells, however, have different gene expression and DNA methylation patterns compared with ES cells and do not contribute to adult chimaeras. We proposed that the incomplete reprogramming might be due to the selection for *Fbx15* expression, and that by using better selection markers, we might be able to generate more ES-cell-like iPS cells. We decided to use *Nanog* as a candidate of such markers.

Although both *Fbx15* and *Nanog* are targets of Oct3/4 and Sox2 (refs 9–11), Nanog is more tightly associated with pluripotency. In contrast to *Fbx15*-null mice and ES cells that barely show abnormal phenotypes[9], disruption of *Nanog* in mice results in loss of the pluripotent epiblast[12]. *Nanog*-null ES cells can be established, but they tend to differentiate spontaneously[12]. Forced expression of *Nanog* renders ES cells independent of leukaemia inhibitory factor (LIF) for self-renewal[12,13] and confers increased reprogramming efficiency after fusion with somatic cells[14]. These results prompted us to propose that if we use *Nanog* as a selection marker, we might be able to obtain iPS cells displaying a greater similarity to ES cells.

Generation of Nanog iPS cells

To establish a selection system for *Nanog* expression, we began by isolating a bacterial artificial chromosome (BAC, ~200 kilobases) containing the mouse *Nanog* gene in its centre. By using recombineering technology[15,16], we inserted a green fluorescent protein (GFP)-internal ribosome entry site (IRES)-puromycin resistance gene (Puro^r) cassette into the 5′ untranslated region (UTR; Fig. 1a). ES cells that had stably incorporated the modified BAC were positive for GFP, but became negative when differentiation was induced (not shown). By introducing these ES cells into blastocysts, we obtained chimaeric mice and then transgenic mice containing the Nanog-GFP-IRES-Puro^r reporter construct. In transgenic mouse blastocysts, GFP was specifically observed in the inner cell mass (Fig. 1b). In 9.5 days post coitum (d.p.c.) embryos, only migrating primordial germ cells (PGCs) showed GFP signal. In 13.5 d.p.c. embryos, GFP was specifically detected in the genital ridges of both sexes. After removing the brain, visceral tissues and genital ridges, we isolated mouse embryonic fibroblasts (MEFs) from 13.5 d.p.c. male embryos. Flow cytometry analyses showed that these MEFs did not contain GFP-positive cells, whereas ~1% of cells isolated from genital ridges showed GFP signals (Fig. 1c).

Next, we introduced the four previously described factors (Oct3/4, Sox2, Klf4 and the c-Myc mutant c-Myc(T58A)) into Nanog-GFP-IRES-Puro^r MEFs cultured on SNL feeder cells with the use of retroviral vectors. Three, five, or seven days after retroviral infection, we started puromycin selection in ES cell medium. GFP-positive cells first became apparent ~7 days after infection. Twelve days after infection, a few hundred colonies appeared, regardless of the timing of puromycin selection (Fig. 2a). By contrast, no colonies emerged from MEFs transfected with mock DNA. Among puromycin-resistant colonies, ~5% were positive for GFP (Fig. 2b). When the puromycin selection was started at 7 days after infection, we obtained the most GFP-positive colonies. Because we used the GFP-IRES-Puro^r cassette, it is unclear why we obtained GFP-negative colonies. With increased concentrations of puromycin, we obtained fewer GFP-negative colonies (Fig. 2c). With any combination of three of the four factors, we did not obtain any GFP-positive colonies (Supplementary Fig. 1).

By continuing cultivation of these GFP-positive colonies, we obtained cells that were morphologically indistinguishable from ES cells (Fig. 2d). These cells also demonstrated ES-like proliferation, with slightly longer doubling times than that of ES cells (Fig. 3a). Subcutaneous transplantation of these cells into nude mice resulted in tumours that consisted of various tissues of all three germ layers,

[1]Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan. [2]CREST, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan.

indicating that these cells are pluripotent (Fig. 3b and Supplementary Fig. 2). We therefore refer to these cells as Nanog iPS cells in the remainder of this manuscript. Induced pluripotent stem cells were established from Fbx15 β-geo MEFs in parallel and are referred to as Fbx15 iPS cells.

Similarity between Nanog iPS cells and ES cells

Polymerase chain reaction with reverse transcription (RT–PCR) showed that Nanog iPS cells expressed most ES cell marker genes, including *Nanog*, at higher and more consistent levels compared with Fbx15 iPS cells (Fig. 4a). DNA microarray analyses confirmed that Nanog iPS cells had greater ES-cell-like gene expression compared with Fbx15 iPS cells (Fig. 4b). The expression level of *Rex1* (also called *Zfp42*) in Nanog iPS cells was higher compared with Fbx15 iPS cells, but still lower than in ES cells. Thus, Nanog iPS cells show greater gene expression similarity to ES cells (without being identical) than do Fbx15 iPS cells.

RT–PCR showed that Nanog iPS cells have significantly lower expression levels of the four transgenes than Fbx15 iPS cells (Fig. 4c). Real-time PCR confirmed that transgene expression was very low in Nanog iPS cells (Supplementary Fig. 4a–d). In contrast, Southern blot analyses showed similar copy numbers of retroviral integration in Nanog iPS cells and Fbx15 iPS cells (Supplementary Fig. 5). These data indicate that retroviral transgene expression is largely silenced in Nanog iPS cells, as has been shown in ES cells[17]. The expression levels of the transgenes are reversely correlated with *Dnmt3a2* expression, suggesting that *de novo* methyltransferase[18] may be involved in the retroviral silencing observed in iPS cells (Supplementary Fig. 6).

Bisulphite genomic sequencing analyses also revealed similarities between Nanog iPS cells and ES cells (Fig. 5). The promoter regions of *Nanog*, *Oct3/4* and *Fbx15* were largely unmethylated in Nanog iPS cells. This is in marked contrast to Fbx15 iPS cells in which the promoters of *Nanog* and *Oct3/4* were only partially unmethylated[8]. Differentially methylated regions of imprinting genes *H19* and *Igf2r* were partially methylated in Nanog iPS cells. During PGC development, imprinting is erased by 12.5 d.p.c.[19–21]. The loss of imprinting is maintained in embryonic germ cells derived from 12.5 d.p.c. PGCs[22] and cloned embryos derived from 12.5–16.5 d.p.c. PGCs[23,24]. ES cells, by contrast, showed normal imprinting patterns[25]. Thus, Nanog iPS cells show greater similarity in the methylation patterns of imprinting genes to ES cells than to embryonic germ cells.

Simple sequence length polymorphism (SSLP) analyses showed that Nanog iPS cells are largely of the DBA background but also have some contribution from the C57BL/6 and 129S4 backgrounds (Supplementary Fig. 3). This result is consistent with the genetic background of the MEFs, which was 75% DBA, 12.5% C57BL/6

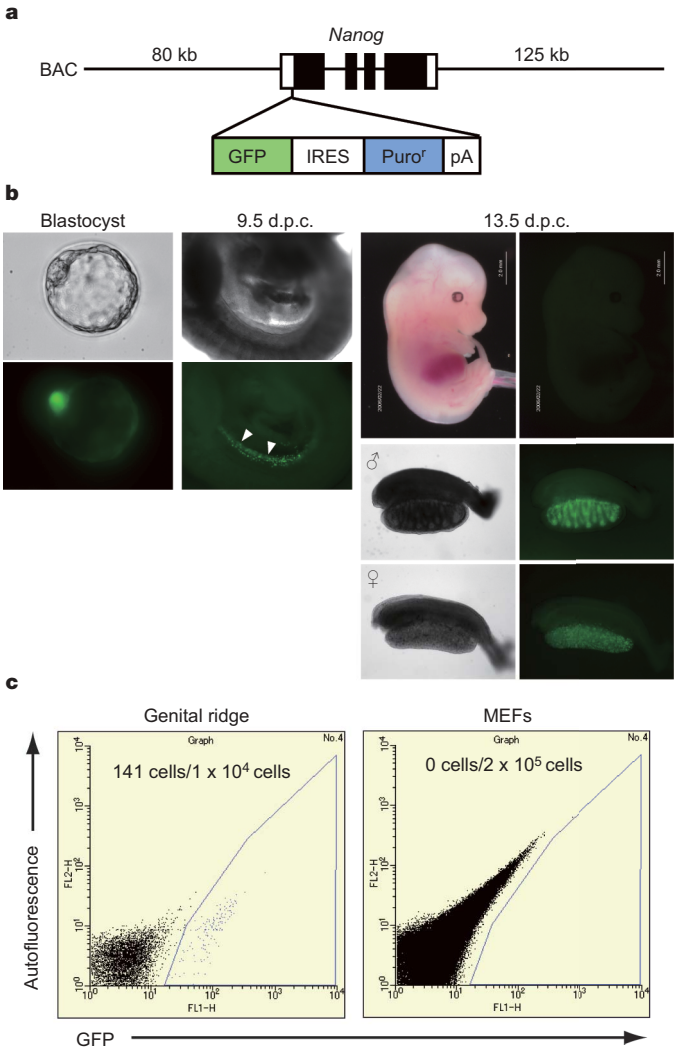

Figure 1 | Nanog-GFP-IRES-Puro^r transgenic mice. **a**, Modified BAC construct. White boxes indicate the 5′ and 3′ UTRs of the mouse *Nanog* gene. Black boxes indicate the open reading frame. **b**, GFP expression in Nanog-GFP transgenic mouse embryos. Whole embryos (top panels) and isolated genital ridges (bottom panels) from 13.5 d.p.c. mice are shown. **c**, Histogram showing GFP fluorescence in cells isolated from genital ridges of a 13.5 d.p.c. Nanog-GFP transgenic mouse embryo (left) or in MEFs isolated from the same embryo (right).

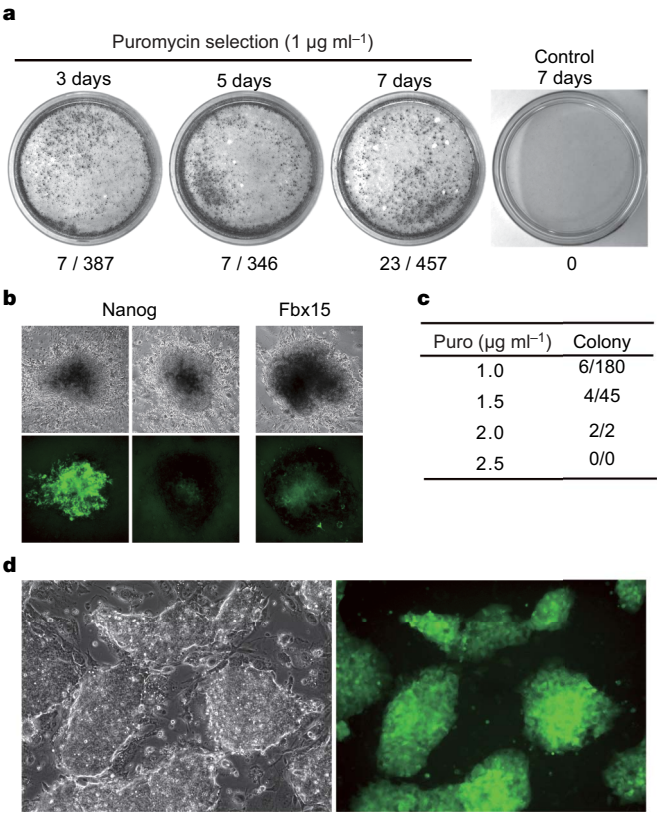

Figure 2 | Generation of iPS cells from MEFs of Nanog-GFP-IRES-Puro^r transgenic mice. **a**, Puromycin-resistant colonies. Puromycin selection was initiated at 3, 5, or 7 days after retroviral transduction. Numbers indicate GFP-positive colonies/total colonies. **b**, GFP fluorescence in resulting colonies. Phase contrast (top row) and fluorescence (bottom row) micrographs are shown. iPS cells were also generated from Fbx15-βgeo knockin MEFs. **c**, Effect of increasing concentrations of puromycin. Numbers of GFP-positive colonies/total colonies are shown on the right. **d**, Morphology of established Nanog iPS cells (clone 20D17). Phase contrast (left) and fluorescence (right) micrographs are shown.

and 12.5% 129S4. This result also confirms that Nanog iPS cells are not a contamination of ES cells that exists in our laboratory, which are either pure 129S4 or C57BL/6.

We next compared the stability of Nanog iPS cells and Fbx15 iPS cells (Supplementary Fig. 7). Cells were cultivated in the presence of the selection drug for up to 22–26 passages. Morphologically, we did not observe significant changes over the long-term culture course. However, RT–PCR showed that Fbx15 iPS cells lost the expression of ES cell marker genes after prolonged culture. By contrast, Nanog iPS cells maintained relatively high expression levels of the ES cell marker genes. These data demonstrate that Nanog iPS cells are more stable than Fbx15 iPS cells.

We also compared the induction efficiency of Nanog iPS cells and Fbx15 iPS cells. In independent experiments, we obtained 4–125 GFP-positive colonies from 8×10^5 Nanog-reporter MEFs transfected with the four transcription factors. Because ~50% of transfected MEFs are supposed to express all four factors[8], the induction efficiency is approximately 0.001–0.03%. In contrast, from the same number of Fbx15-reporter MEFs, we obtained 47–1,800 G418-resistant colonies, with the induction efficiency of approximately 0.01–0.5%. Thus, the efficiency of Nanog iPS cell induction is approximately one-tenth that of Fbx15 iPS cells.

We then compared the responses of Nanog iPS cells and Fbx15 iPS cells to LIF or retinoic acid (Supplementary Fig. 8). As we have shown previously[8], Fbx15 iPS cells do not remain undifferentiated when cultured without feeder cells, even in the presence of LIF. Furthermore, Fbx15 iPS cells formed compact colonies when cultured without feeder cells in the presence of retinoic acid. In contrast, LIF maintained the undifferentiated state of Nanog iPS cells cultured without feeder cells. Retinoic acid induced the differentiation of Nanog iPS cells. Thus, Nanog iPS cells are similar to ES cells in their response to LIF and retinoic acid.

Initially we used the T58A mutant of c-Myc to induce Nanog iPS cells. We also tested wild-type c-Myc for Nanog iPS cell induction. We obtained a similar number of colonies with both wild-type c-Myc and the T58A mutant. Nanog iPS cells established with wild-type c-Myc were indistinguishable from those established with the T58A mutant with regards to morphology, gene expression (analysed via microarrays), teratoma formation (Supplementary Fig. 2) and stability under puromycin selection (Supplementary Fig. 9). Without puromycin selection, Nanog iPS cells induced by wild-type c-Myc were more stable (Supplementary Fig. 9).

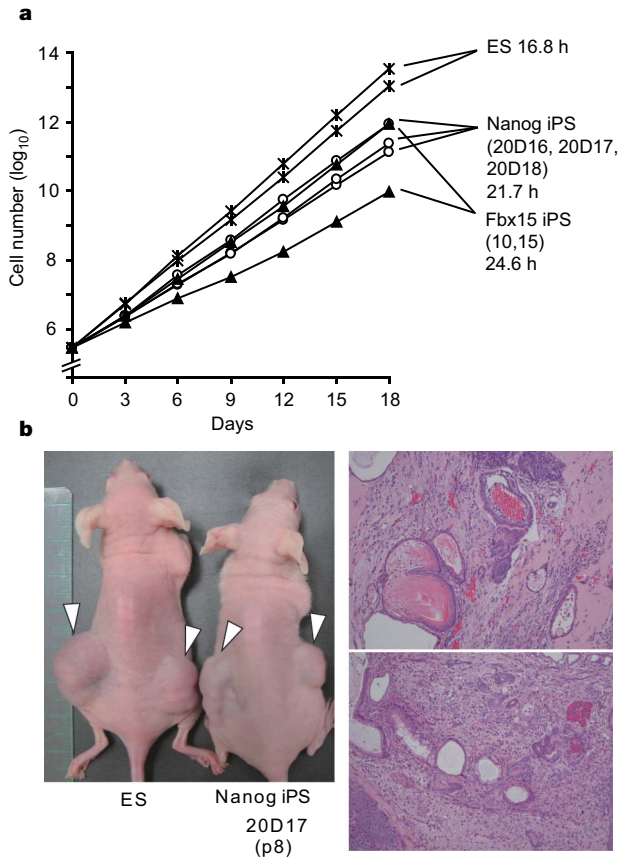

Figure 3 | Characterization of Nanog iPS cells. **a**, Proliferation. ES cells, Nanog iPS cells (clones 20D16, 20D17 and 20D18) and Fbx15 iPS cells (clones 10 and 15) were passaged every 3 days (3×10^5 cells per each well of a 6-well plate). Calculated doubling times are indicated. **b**, Teratomas. ES cells or Nanog iPS cells (clone 20D17, 1×10^6 cells) were subcutaneously transplanted into nude mice. After 8 weeks, teratomas were photographed (left) and analysed histologically with haematoxylin and eosin staining (right).

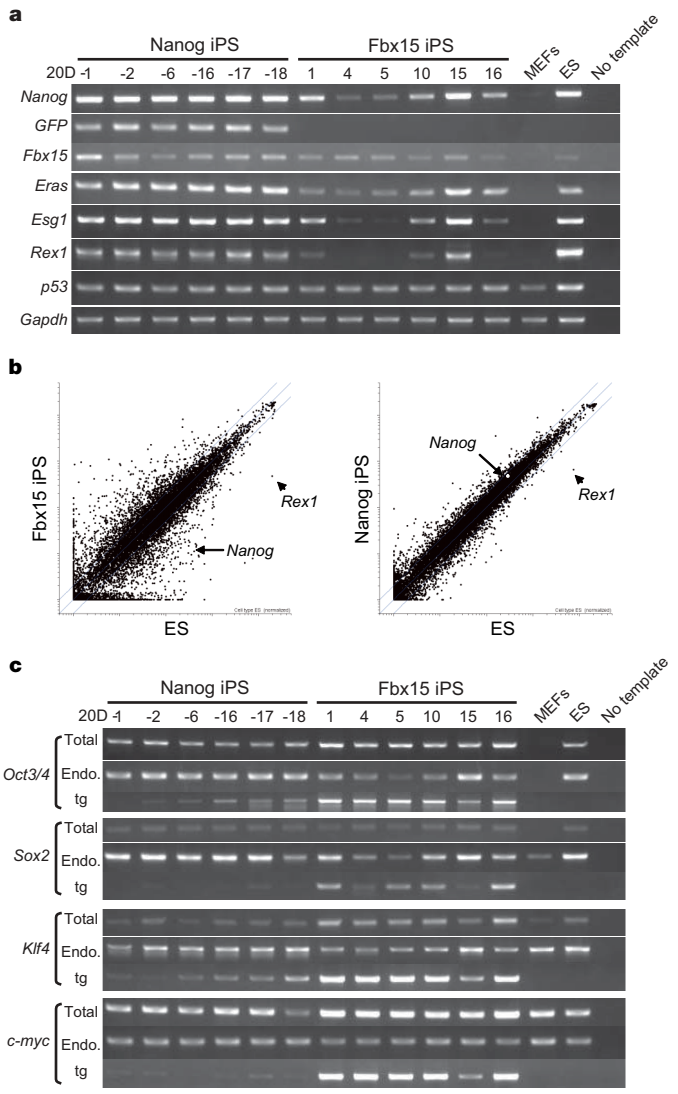

Figure 4 | Gene expression in Nanog iPS cells. **a**, RT–PCR. Total RNA was isolated from six clones of Nanog iPS cells (clones 20D1, 20D2, 20D6, 20D16, 20D17 and 20D18), six clones of Fbx15 iPS cells (clones 1, 4, 5, 10, 15 and 16), MEFs and ES cells. **b**, Scatter plots showing comparison of global gene expression between ES cells and Nanog iPS cells (right), and between ES cells and Fbx15 iPS cells (left), as determined by DNA microarrays. **c**, Expression levels of the four transcription factors. Total RNA was isolated from six clones of Nanog iPS cells (clones 20D1, 20D2, 20D6, 20D16, 20D17 and 20D18), six clones of Fbx15 iPS cells (clones 1, 4, 5, 10, 15 and 16), MEFs and ES cells. RT–PCR analyses were performed with primers that amplified the coding regions of the four factors (Total), endogenous transcripts only (Endo.), and transgene transcripts only (tg).

Germline chimaeras from Nanog iPS cells

We next examined the ability of Nanog iPS cells to produce adult chimaeras. We injected 15–20 male Nanog iPS cells (five clones with the T58A mutant and three with wild-type c-Myc) into C57BL/6-derived blastocysts, which we then transplanted into the uteri of pseudo-pregnant mice. We obtained adult chimaeras from seven clones (four clones with the T58A mutant and three with wild-type c-Myc) as determined by coat colour (Fig. 6a and Supplementary Table 1). SSLP analyses showed that Nanog iPS cells contributed to various organs, with the level of chimaerism ranging from 10% to 90%. Chimeras from clone 20D17 showed highest iPS cell contribution in the testes. From clone 20D18, we obtained only a few non-chimaeric pups from infected blastocysts; thus, whether this clone has competency for producing adult chimaeras remained to be determined. These data demonstrate that most Nanog iPS clones are competent for adult chimaeric mice.

We then crossed three of the chimaeras from clone 20D17—for which the highest iPS cell contribution was in the testes—with C57BL/6 females. Whereas all F_1 mice showed black coat colour, all contained retroviral integration of the four transcription factors and approximately half contained the GFP-IRES-Puro^r cassette (Fig. 6b), indicating germline transmission. Furthermore, approximately half of the F_2 mice born from F_1 intercrosses showed agouti coat colour, confirming germline transmission of Nanog-iPS-20D17 (Fig. 6c).

We also examined germline competency for two other clones that produced adult chimaeras. In one chimaeric mouse from Nanog-iPS-38C2 cell line, PCR analysis detected iPS cell contribution in isolated spermatozoa (Fig. 6d), suggesting that germline competency is not confined to clone 20D17. However, the iPS cell contribution to sperm of clone 38C2 is much smaller than that of clone 20D17, and no iPS-cell-derived offspring were found for 119 mice born from the cross between the 38C2 chimaera and C57BL/6 female mice. Most male mice with a high degree of chimaerism from the Nanog-iPS-38D2 cell line showed small testes and aspermatogenesis (Supplementary Fig. 13). The testes of some chimaeras from Nanog-iPS-38D2 contained mature sperm, but no iPS cell contribution was detected by PCR (Fig. 6d).

Tumour formation by *c-myc* reactivation

Out of 121 F_1 mice (aged 8–41 weeks) derived from the Nanog-iPS-20D17 cell line, 24 died or were killed because of weakness, wheezing or paralysis. Necropsy of 17 mice identified neck tumours (Supplementary Fig. 10) in 13 mice and other tumours in five mice, including two mice with neck tumours. Histological examination of one neck tumour showed that it was a ganglioneuroblastoma with follicular carcinoma of the thyroid gland (not shown). In these tumours, retroviral expression of *c-myc*, but not *Oct3/4*, *Sox2*, or *Klf4*, is reactivated (Supplementary Fig. 11). In contrast, transgene expression of all four transcription factors remained low in normal tissues, except for *c-myc* in muscle in one mouse (Supplementary Fig. 12). These data indicate that reactivation of *c-myc* retrovirus is attributable to tumour formation.

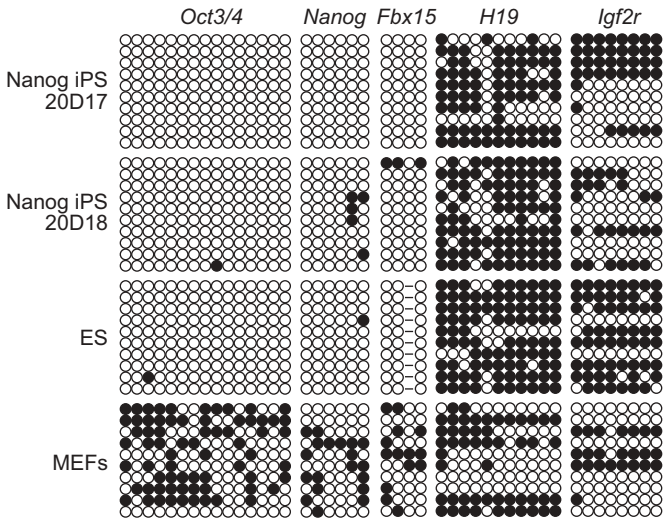

Figure 5 | DNA methylation of ES-cell-specific genes and imprinting genes. White circles indicate unmethylated CpG dinucleotides, whereas black circles indicate methylated CpG dinucleotides.

Discussion

Our results demonstrate that Nanog selection allows the generation of high-quality iPS cells that are comparable to ES cells in morphology, proliferation, teratoma formation, gene expression and competency for adult chimaeras. Nearly all Nanog iPS clones showed these properties, indicating that Nanog is a major determinant of quality in cellular pluripotency. However, germline competence was variable among Nanog iPS clones, indicating the existence of other important determinants of germline competency in addition to Nanog. The high quality of Nanog iPS cells underscores the possibility of using this technology to generate patient-specific pluripotent stem cells. In a separate study, we found that germline-competent iPS cells can also be obtained from adult mouse somatic cells (T. Aoi and S.Y., unpublished data). The current study, however, also reveals that reactivation of *c-myc* retrovirus may result in tumour formation. There may be ways to overcome this problem. Strong silencing of the four retroviruses in Nanog iPS cells indicates that they are only required for the induction, but not the maintenance, of pluripotency. Therefore, the retrovirus-mediated system might be

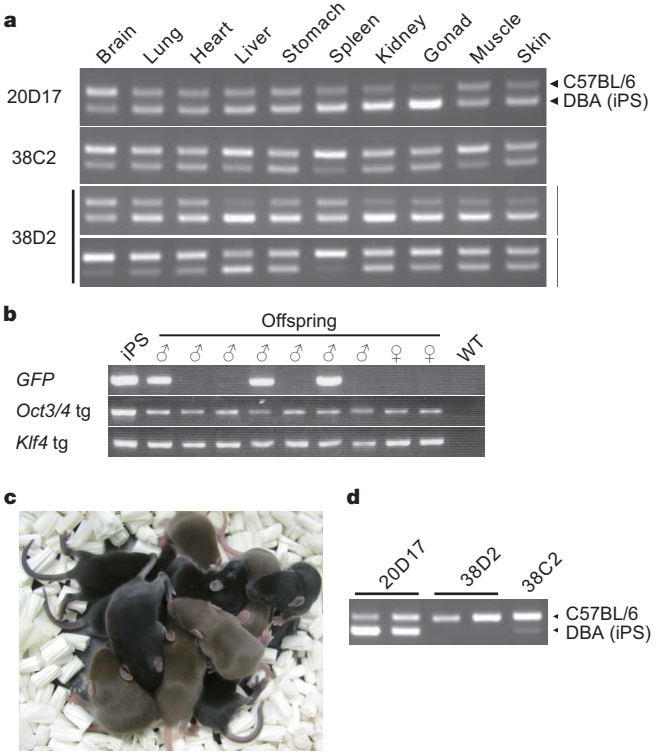

Figure 6 | Germline chimaeras from Nanog iPS cells. a, Tissue distribution of iPS cells in chimaeras. Genomic DNA was isolated from the indicated organs of chimaeras derived from three Nanog iPS cell clones (20D17, 38C2 and 38D2). SSLP analyses were performed for D6Mit15. **b**, PCR analyses showing the presence of the GFP cassette and retroviral transgenes in F_1 mice obtained from the intercross between a chimaeric male and a C57BL/6 female. **c**, Coat colours of F_2 mice obtained from F_1 intercrosses. **d**, Sperm contribution of iPS cells in chimaeric mice. Spermatozoa were isolated from the epididymides of chimaeric mice derived from three Nanog iPS cell clones (20D17, 38D2 and 38C2). iPS cell contribution was determined by SSLP of D6Mit15.

eventually replaced by transient expression, such as the adenovirus-mediated system. Alternatively, high-throughput screening of chemical libraries might identify small molecules that can replace the four genes. These are crucial research areas in order to apply iPS cells to regenerative medicine.

We found that the efficiency of Nanog iPS cell induction is less than 0.1%. The low efficiency suggests that the origin of iPS cells might be rare stem cells co-existing in MEF culture. Alternatively, activation of additional genes by retroviral integration might be required for iPS cell generation in addition to the four transcription factors. This is relevant to the fact that we have been able to obtain iPS cells only with retroviral transduction. Identification of such factor(s) may lead to the generation of iPS cells with higher efficiency, and without the need for retroviruses.

METHODS SUMMARY

To generate Nanog-reporter mice, we isolated a BAC clone containing the mouse *Nanog* gene in its centre. By using the RED/ET recombination technique (Gene Bridges), we inserted a GFP-IRES-Puro^r cassette into the 5′ UTR of the mouse *Nanog* gene. We introduced the modified BAC into RF8 ES cells by electroporation[26]. We then microinjected transgenic ES cells into C57BL/6 blastocysts to generate Nanog-reporter mice containing the modified BAC. MEFs were isolated from 13.5 d.p.c. male embryos after removing genital ridges. Generation of Nanog iPS cells was performed as described[8], except that puromycin was used instead of G418 as a selection antibiotic. Retroviruses (pMXs) were generated with Plat-E packaging cells[27]. RF8 ES cells[26] and iPS cells were cultured on SNL feeder cells[28]. Analyses of iPS cells, such as RT–PCR, real-time PCR, bisulphite genomic sequencing, SSLP analyses, DNA microarrays, teratoma formation, and microinjection into C57BL/6 blastocysts, were performed as described[8]. Contribution of iPS cells in chimaeric mice was determined by PCR for the SSLP marker D6Mit15.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 6 February; accepted 22 May 2007.
Published online 6 June 2007.

1. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147 (1998).
2. Hochedlinger, K. & Jaenisch, R. Nuclear reprogramming and pluripotency. *Nature* **441,** 1061–1067 (2006).
3. Niwa, H., Miyazaki, J. & Smith, A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet.* **24,** 372–376 (2000).
4. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95,** 379–391 (1998).
5. Avilion, A. A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* **17,** 126–140 (2003).
6. Cartwright, P. *et al.* LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* **132,** 885–896 (2005).
7. Li, Y. *et al.* Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood* **105,** 635–637 (2005).
8. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126,** 663–676 (2006).
9. Tokuzawa, Y. *et al.* Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. *Mol. Cell. Biol.* **23,** 2699–2708 (2003).
10. Kuroda, T. *et al.* Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol. Cell. Biol.* **25,** 2475–2485 (2005).
11. Rodda, D. J. *et al.* Transcriptional regulation of Nanog by OCT4 and SOX2. *J. Biol. Chem.* **280,** 24731–24737 (2005).
12. Mitsui, K. *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113,** 631–642 (2003).
13. Chambers, I. *et al.* Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113,** 643–655 (2003).
14. Silva, J., Chambers, I., Pollard, S. & Smith, A. Nanog promotes transfer of pluripotency after cell fusion. *Nature* **441,** 997–1001 (2006).
15. Copeland, N. G., Jenkins, N. A. & Court, D. L. Recombineering: a powerful new tool for mouse functional genomics. *Nature Rev. Genet.* **2,** 769–779 (2001).
16. Testa, G. *et al.* Engineering the mouse genome with bacterial artificial chromosomes to create multipurpose alleles. *Nature Biotechnol.* **21,** 443–447 (2003).
17. Cherry, S. R., Biniszkiewicz, D., van Parijs, L., Baltimore, D. & Jaenisch, R. Retroviral expression in embryonic stem cells and hematopoietic stem cells. *Mol. Cell. Biol.* **20,** 7419–7426 (2000).
18. Chen, T., Ueda, Y., Xie, S. & Li, E. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. *J. Biol. Chem.* **277,** 38746–38754 (2002).
19. Davis, T. L., Yang, G. J., McCarrey, J. R. & Bartolomei, M. S. The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum. Mol. Genet.* **9,** 2885–2894 (2000).
20. Sato, S., Yoshimizu, T., Sato, E. & Matsui, Y. Erasure of methylation imprinting of Igf2r during mouse primordial germ-cell development. *Mol. Reprod. Dev.* **65,** 41–50 (2003).
21. Brandeis, M. *et al.* The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. *EMBO J.* **12,** 3669–3677 (1993).
22. Labosky, P. A., Barlow, D. P. & Hogan, B. L. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* **120,** 3197–3204 (1994).
23. Kato, Y. *et al.* Developmental potential of mouse primordial germ cells. *Development* **126,** 1823–1832 (1999).
24. Lee, J. *et al.* Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development* **129,** 1807–1817 (2002).
25. Geijsen, N. *et al.* Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427,** 148–154 (2004).
26. Meiner, V. L. *et al.* Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc. Natl Acad. Sci. USA* **93,** 14041–14046 (1996).
27. Morita, S., Kojima, T. & Kitamura, T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* **7,** 1063–1066 (2000).
28. McMahon, A. P. & Bradley, A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62,** 1073–1085 (1990).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank K. Takahashi, M. Nakagawa and T. Aoi for scientific discussion; M. Maeda for histological analyses; M. Narita, J. Iida, H. Miyachi and S. Kitano for technical assistance; and R. Kato, R. Iyama and Y. Ohuchi for administrative assistance. We also thank T. Kitamura for Plat-E cells and pMXs retroviral vectors, and R. Farese for RF8 ES cells. This study was supported in part by a grant from the Uehara Memorial Foundation, the Program for Promotion of Fundamental Studies in Health Sciences of NIBIO, a grant from the Leading Project of MEXT, and Grants-in-Aid for Scientific Research of JSPS and MEXT (to S.Y.). K.O. is a JSPS research fellow.

Author Contributions K.O. conducted most of the experiments in this study. T.I. performed manipulation of mouse embryos to generate Nanog-GFP transgenic mice. T.I. also maintained the mouse lines. S.Y. designed and supervised the study, and prepared the manuscript. S.Y. also performed computer analyses of DNA microarray data.

Author Information The microarray data are deposited in GEO under accession number GSE7841. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.Y. (yamanaka@frontier.kyoto-u.ac.jp).

METHODS

Cell culture. RF8 ES cells (129S4 background)[26] and iPS cells were maintained in ES medium (DMEM containing 15% FCS, 1× NEAA, 1 mM sodium pyruvate, 5.5 mM 2-ME, 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) on feeder layers of mitomycin-C-treated SNL cells into which we had stably incorporated the puromycin-resistance gene. As a source of LIF, we used conditioned medium from Plat-E cell cultures that had been transduced with a LIF-expressing vector. Plat-E cells[27], which were also used to produce retroviruses, were maintained in DMEM containing 10% FCS, 50 units ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 1 μg ml^{-1} puromycin and 10 μg ml^{-1} blasticidin S.

For MEF isolation, we used 13.5 d.p.c. male embryos. After the removal of the head, visceral tissues and gonads, the remaining bodies were washed and dissociated with trypsin. Ten-million cells were plated on each gelatin-coated 100-mm dish and incubated at 37 °C with 5% CO_2. The next day, floating cells were removed by washing with PBS. In this study, MEFs were used within passage 5 to avoid replicative senescence.

BAC modification. A mouse BAC clone containing the *Nanog* gene, RP23-117I23, was purchased from BACPAC Resources. Modification of the BAC was performed using the RED/ET recombination technique (Gene Bridges), which is based on homologous recombination using inducible RecET recombination machinery. The reporter cassette was made by ligating a GFP-IRES-$Puro^r$ fragment with a PGK-hygro-FRT cassette (Gene Bridges) that contains a prokaryotic promoter and hygromycin-resistance gene flanked by FRT sites. Homology arms for the *Nanog* 5′ UTR were attached to both ends of the reporter cassette by PCR amplification using the following primers: BAC-NanogGFP-F (5′-TTTGCATTAGACATTTAACTCTTCTTTCTATGATCTTTCCTTCTAGACACGCCACCATGGTGAGCAAGGGCGAG-3′) and BAC-NanogR (5′-GCGAGGGAAGGGATTTCTGAAAAGGTTTTAGGCAACAACCAAAAAACTCACTGGCAGTTTATGGCGGGCGTCCT-3′). *Escherichia coli* carrying the BAC was transformed with a RED/ET expression plasmid, pSC101-BAD-gbaA, and recombination with the reporter cassette was subsequently induced. Successfully recombined colonies were identified by screening for hygromycin resistance followed by PCR analysis to ensure homologous recombination. The hygromycin cassette was excised by transformation into a Flp-recombinase expression bacterium, 294-Flp (Gene Bridges).

Establishment of Nanog-reporter mice. The modified BAC was linearized by *Not*I digestion, and 10 μg of the DNA was introduced into RF8 ES cells by electroporation[26]. After 2 days, selection was started with 1.5 μg ml^{-1} puromycin. Resistant colonies were picked after 9 days of selection. Four genomic integrated clones were used for blastocyst injection, and we established two lines of Nanog reporter mice: 2A2 and 2C1. Both mice exhibited the same expression pattern. Mice from the 2A2 line were used for iPS cell induction.

iPS cell induction. iPS induction was performed as described previously[8] with some modifications. Briefly, MEFs were isolated from 13.5 d.p.c. embryos from Nanog-reporter male mice (50% DBA, 25% C57BL/6 and 25% 129S4) and female wild-type mice (DBA). Plat-E cells were seeded at 8×10^6 cells per 100-mm dish. On the next day, 9 μg of pMXs-based retroviral vectors for *Oct3/4*, *Sox2*, *Klf4*, or *c-Myc* were independently introduced into Plat-E cells using 27 μl of FuGENE 6 transfection reagent. After 24 h, the medium was replaced with 10 ml of DMEM containing 10% FCS. MEFs were seeded at 8×10^5 cells per 100-mm dish covered by feeder cells. On the next day, virus-containing supernatants from these Plat-E cultures were recovered and filtered through a 0.45-μm cellulose acetate filter. Equal volumes of the supernatants were mixed and supplemented with polybrene at the final concentration of 4 μg ml^{-1}. MEFs were incubated in the virus/polybrene-containing supernatants for 24 h. Three days after infection, the medium was changed with ES medium supplemented with LIF. For Fbx15 iPS cell selection, we added G418 (Geneticin from Invitrogen) at a final concentration of 0.3 mg ml^{-1}. For Nanog iPS cells, we added puromycin (Sigma) at a final concentration of 1.5 μg ml^{-1}, unless indicated otherwise. Established iPS cells were maintained in the presence of the corresponding selection drug. Teratoma formation, RT–PCR analysis, Bisulphite sequence and SSLP analysis were performed as previously described[8].

DNA microarray. Total RNA from *Fbx15*-null MEFs (duplicate), *Fbx15*-null ES cells (duplicate), Fbx15 iPS cells (clone MEF4-7[8], duplicate), or Nanog iPS cells (clones 20D -2, 16, 17, and 18) were labelled with Cy3. Samples were hybridized to a Mouse Oligo Microarray (Agilent) according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analysed using GeneSprings GX software (Agilent). We excluded genes for which their value fluctuated more than twofold between duplicated analyses.