LETTERS

iPS cells produce viable mice through tetraploid complementation

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Since the initial description of induced pluripotent stem (iPS) cells created by forced expression of four transcription factors in mouse fibroblasts, the technique has been used to generate embryonic stem (ES)-cell-like pluripotent cells from a variety of cell types in other species, including primates and rat[1–6]. It has become a popular means to reprogram somatic genomes into an embryonic-like pluripotent state, and a preferred alternative to somatic-cell nuclear transfer and somatic-cell fusion with ES cells[7,8]. However, iPS cell reprogramming remains slow and inefficient. Notably, no live animals have been produced by the most stringent tetraploid complementation assay, indicative of a failure to create fully pluripotent cells. Here we report the generation of several iPS cell lines that are capable of generating viable, fertile live-born progeny by tetraploid complementation. These iPS cells maintain a pluripotent potential that is very close to ES cells generated from *in vivo* or nuclear transfer embryos. We demonstrate the practicality of using iPS cells as useful tools for the characterization of cellular reprogramming and developmental potency, and confirm that iPS cells can attain true pluripotency that is similar to that of ES cells.

Several studies have shown that iPS cells resemble ES cells in morphology, gene expression profiles, and the epigenetic status of several pluripotency markers[9,10]. Notably, iPS cells have maintained their ability to generate chimaeras that are competent for germline transmission[9–14]. Their therapeutic potential is thought to be similar to that of ES cells in several disease mouse models[15,16]. Despite the promise this technique holds for the development of patient-specific cell therapy, considerable technical barriers remain that must be overcome for application as a therapeutic intervention. To reduce the potential oncogenic effect of genomic integration of viral vectors, different combinations or reduced numbers of factors have been introduced, as well as the use of alternative delivery methods and agents[11,13,17–21]. However, one of the remaining technical hurdles in the reprogramming process is the slow and inefficient induction of iPS cells, especially those with a complete developmental potential capable of producing live-born offspring. Tetraploid complementation is considered the most stringent test for pluripotency and developmental potency, as any viable live-born animals resulting from the injection of diploid ES or iPS cells to create the tetraploid (4N) embryos (blastocysts) will be from the diploid donor cells. The tetraploid host blastocyst primarily contributes to the extra-embryonic lineages and not to the embryo proper[22,23]. Unlike pluripotent ES cells generated from *in vivo* or somatic-cell nuclear transfer (SCNT)-produced embryos, all previous studies have failed to produce viable offspring from iPS cells through tetraploid complementation[9,13,24].

Here we report the generation of 37 iPS cell lines that not only express the correct pluripotency markers by *in vitro* assays, but many are also able to produce chimaeric mice with germline transmission by blastocyst injection. Three of these lines produced viable, live-born offspring by tetraploid complementation.

We infected mouse embryonic fibroblast (MEF) cells expressing an Oct4 (also known as Pou5f1)-enhanced green fluorescent protein (GFP) reporter with the four 'Yamanaka factors'[1,25]: pMXs-Oct4, Sox2, Klf4 and c-Myc, and cultured the cells as previously reported[25] with modifications such that knockout serum replacement (KOSR)[26,27] was substituted for fetal bovine serum (FBS) for iPS cell derivation. At day 10, alkaline phosphatase$^+$ and GFP$^+$ colonies were detected and iPS cell lines were derived at days 14, 20 and 36 (Fig. 1a). From several experimental runs, 31 GFP$^+$ colonies were obtained and yielded stable cell lines (Supplementary Table 1). In addition to the Oct4–GFP MEF cells from a B6D2F1 genetic background, six iPS cell lines from MEF cells with a C57 × 129S2 background were also generated (IP14D-101-106). Because our laboratory does not maintain any ES or SCNT-ES cell lines of this genetic background, these iPS cells could only result from induced fibroblasts (Table 1).

Pluripotency markers such as Oct4, Nanog and SSEA1 were expressed (Fig. 1b), and the cell lines were predominantly diploid with normal 40 chromosome karyotypes (Supplementary Fig. 1). Bisulphite sequencing showed demethylation of *Oct4* and *Nanog* promoters in three iPS cell lines tested compared to the parental Oct4–GFP MEF cells, a pattern similar to that from normal ES cells (Supplementary Fig. 2) reflecting the epigenetic remodelling that occurred during reprogramming. Teratomas with all three germ layers determined by histological analyses were observed from iPS cells injected into severe combined immunodeficient (SCID) mice (Fig. 1c), indicating a considerable degree of pluripotency. Quantitative reverse transcription–polymerase chain reaction (RT–PCR; Supplementary Fig. 3) demonstrated that the transgenes were almost completely silenced in the iPS cell lines tested (IP36D, IP20D and IP14D), similar to previous reports[1,9] suggesting that the maintenance of iPS cell lines mainly requires the endogenous genes of these four factors.

To test pluripotency further, we randomly selected one or two cell lines from each of the experimental runs, and injected them into normal CD-1 blastocysts that were transferred to CD-1 pseudopregnant recipient females. As shown in Table 1, IP36D and IP20D cell lines produced many live mice with 5–80% chimaerism, qualitatively assessed by coat colour. Germline transmission was noted in two of the four lines. Five of the thirteen IP14D iPS cell lines (four with B6D2F1 and one with a C57 × 129S2 background) were randomly selected, and all resulted in live chimaeric mice with 10–95% chimaerism. From

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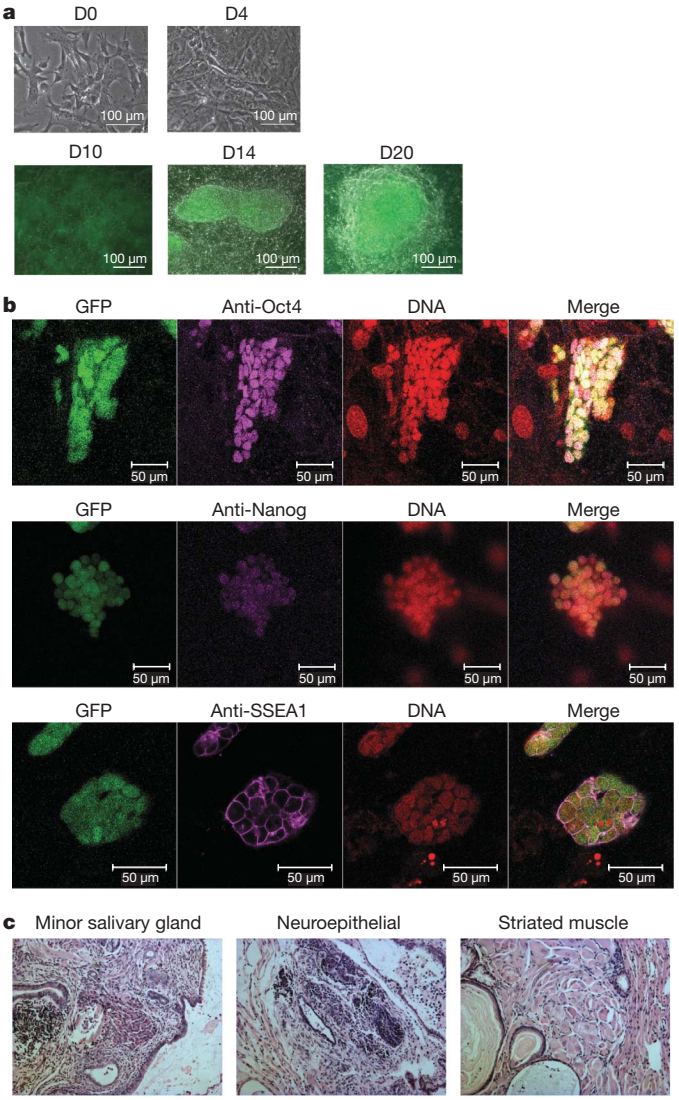

Figure 1 | Characterization of the iPS cells generated in 20% knockout serum replacement culture systems. **a**, Cell morphology. Top, Oct4–GFP MEF cells at day (D) 0 (before viral infection) and day 4 (just before replating onto MEF feeder cells). Bottom, morphology of GFP^+ cells at days 10, 14 and 20. **b**, Immunostaining for pluripotency markers in Oct4–GFP iPS cells. Positive GFP fluorescence (green) and Oct4, Nanog and SSEA1 (purple) were observed. DNA was stained by propidium iodide (red). Shown are examples from the IP20D-3 line. The other two cell lines analysed also gave the same pattern. **c**, Teratoma formation. Sections were stained with haematoxylin and eosin. Shown are tissues representative of all three germ layers: minor salivary gland (endoderm), neuroepithelial (ectoderm) and striated muscle (mesoderm). Original magnification, ×100.

this source, three of the five lines were germline-transmitted to the next generation (Fig. 2). Thus, our iPS cell lines are competent for germline transmission with higher efficiency in the IP14D group compared to IP20D and IP36D.

Until now, the developmental potential of iPS cells could not be fully evaluated because embryos generated from iPS cells using tetraploid complementation ('iPS 4N-comp') were viable only into the later stages of gestation[9,13,24], demonstrating that complete pluripotency had not been attained. When we injected our iPS cells into tetraploid CD-1 blastocysts (white coat), we observed complete development potential resulting in the birth of live pups. Different iPS cell lines were not equally successful in producing viable offspring, although all were competent for germline transmission through chimaeric mice. Cell lines IP36D-3, IP20D-3 and IP20D-19 showed early termination of fetal development at embryonic day (E) 13.5 and E15.5 (Table 1). There seems to be a developmental advantage for the cell line derived at day 20 compared to day 36 (8.8% of IP20D-19 versus 1.7% of IP36D-3 reached E13.5). One IP20D-3 embryo developed normally until E15.5 (Table 1). Figure 2a and b show iPS 4N-comp embryos with normal embryonic morphology collected at E9.5 and E13.5. In contrast, when we injected cells from IP14D-1 (derived from GFP^+ colonies at day 14) into tetraploid blastocysts, 22 live-born pups were obtained (3.5%), as well as four embryos (0.6%) that developed to E17.5. Two other IP14D lines with B6D2F1 and C57 × 129S2 backgrounds (IP14D-6 and IP14D-101, respectively) were able to produce live iPS 4N-comp pups. The rate of iPS 4N-comp animal production is similar to the rate from normal ES cells in our laboratory (Table 1). The IP14D 4N-comp pups have survived from 2 days to almost 9 months so far. The black mouse in Fig. 2f represents one of the live iPS 4N-comp mice at 15 weeks. Note the uniform black coat of this 'all-iPS' mouse, resembling the coat colour of the original line of the Oct4–GFP MEF cells (B6D2F1) from which it was derived.

To identify clearly the lineage of the cells and the 4N-comp mice, simple sequence length polymorphism (SSLP) analyses followed by PCR for various marker genes were performed. The iPS 4N-comp mice differed from the CD-1 mice at markers consistent with a B6D2F1 lineage or the C57 × 129S2 origin, but had exactly the same profiles as the iPS cell lines from which they were derived (Fig. 2c). This was confirmed by PCR analysis of unique microsatellites (Fig. 2d). The iPS cell lines, 2N chimaeric mouse and offspring, iPS 4N-comp mice and their progeny mated to CD-1 females all yielded the expected inheritance patterns, confirming that the IP14D-1 lineage of the iPS 4N-comp adults is distinct from the CD-1 mouse. Furthermore, Southern blot analysis and reverse PCR results independently confirmed that the viral integration patterns in the iPS 4N-comp mice are identical to the three iPS cell lines from which they originated (Supplementary Figs 4 and 5).

To study the embryonic development of offspring from iPS 4N-comp mice, a 7-week-old male mouse carrying Oct4–GFP was mated to a CD-1 female. The iPS mouse was capable of impregnating the dam and producing viable preimplantation stage embryos with normal morphology and without developmental delay. GFP^+

Table 1 | Developmental efficiency of embryos produced

Cell line	2N injection				4N injection (%)			
	Injected blastocysts	Live chimaeras	Chimaerism (%)	Germ line	Injected blastocysts	Embryos arrested at E10.5–E13.5	Embryos arrested at E15.5–E17.5	Live pups
IP14D-1	135	6	20–95	Yes	624	7 (1.1)	4 (0.6)	22 (3.5)
IP14D-6	97	10	20–90	Yes	43	–	–	1 (2.3)
IP14D-101	360	18	10–90	Yes	181	15 (8.3)	–	4 (2.2)
IP20D-3	236	14	20–80	Yes	204	6 (2.9)	2 (1.0)	0
IP20D-19	48	4	10–70	Yes	273	24 (8.8)	–	0
IP36D-3	233	6	5–70	Not yet	229	4 (1.7)	0	0
Control (ES1)	NT	–	–	–	100	–	–	3 (3.0)
Control (CL11)	NT	–	–	–	51	5 (9.8)	–	1 (2.0)

Embryos were produced by blastocyst injection (2N) and tetraploid complementation (4N). IP14D-101 has a genetic background of C57 × 129S2, whereas the rest are from C57 × DBA F_1 (B6D2F1). NT, not tested.

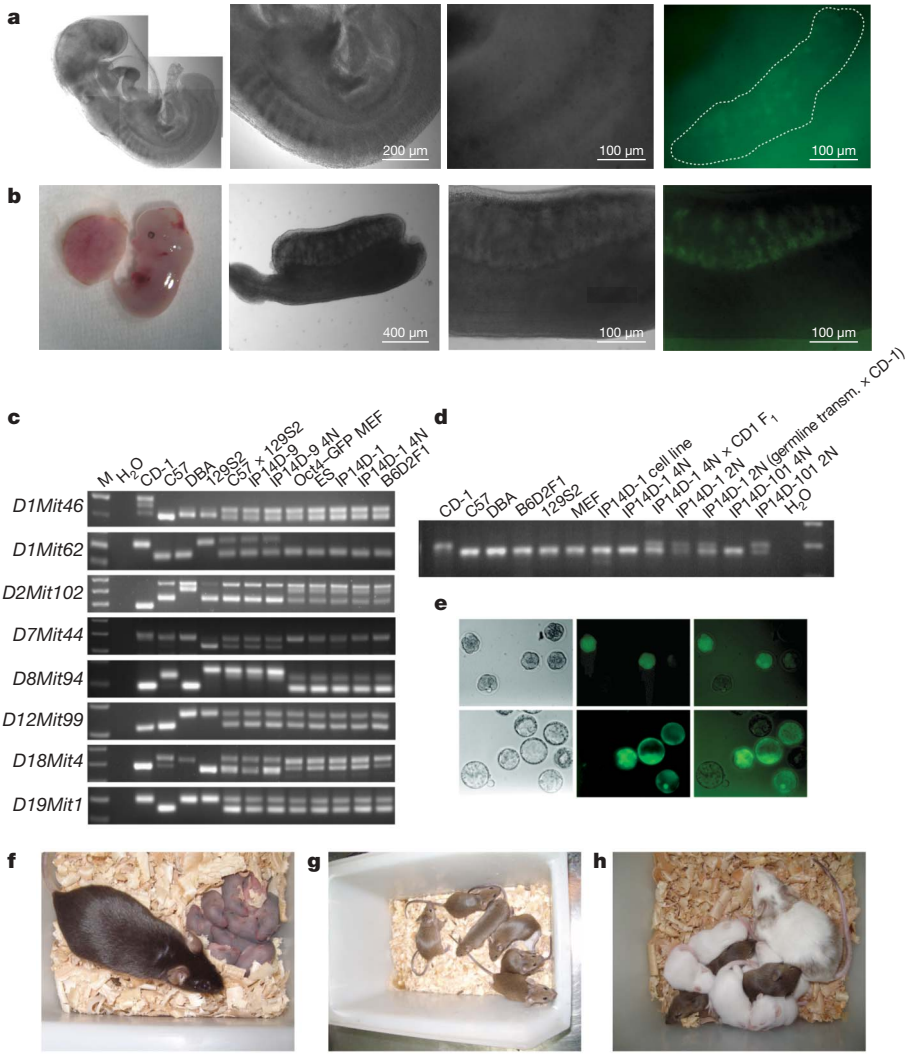

Figure 2 | *In vivo* developmental potential of iPS cell lines generated by tetraploid complementation. Ten to fifteen iPS cells (B6D2F1, black coat colour) were injected into each CD-1 (white coat colour) tetraploid blastocyst electrofused at the two-cell stage, and then transferred into pseudopregnant female recipients. Embryos derived from tetraploid blastocyst injection were dissected on E9.5, E13.5 and on the day of birth (E19.5). **a**, A live E9.5 IP36D-3-derived embryo (left) with 18 somites (middle left). Primordial germ cells migrate along hind-gut mesentery (middle right). Oct4–GFP-positive migrating primordial germ cells are shown (right). **b**, A live E13.5 embryo (left) with placenta derived from IP20D-3. Male gonad and mesonephros of fetus (middle left) are shown. Oct4–GFP is positive (right) in male gonadal germ cells in fetal testes (middle right). **c**, SSLP analysis for lineage identification covers markers from different chromosomes, and the 4N mice showed a polymorphic pattern similar to that from the parental MEF cells originating from B6D2F1 or C57 × 129S2 chimaeras, and different from the CD-1 tetraploid blastocyst donor. M denotes molecular mass marker. **d**, Microsatellite markers detect differences between CD-1 and parental patterns of the inbred C57 and DBA or a hybrid C57 × 129S2 strain, differentiating DNA from the iPS cell lines and the corresponding 4N-comp mouse, from a 2N chimaeric mouse or the 4N-comp × CD-1 F_1 offspring. **e**, Oct4–GFP positive morula (top) and blastocyst (bottom) flushed from CD-1 mated with an IP14D-1 4N mouse. Phase (left), fluorescence (middle) and merged (right) images are shown. Original magnification, ×200. **f**, A fifteen-week-old iPS-tetraploid male mouse derived from IP14D-1, with a uniformly black coat of the B6D2F1 strain from which Oct4–GFP MEF cells originate. The pups lying next to it are its F_1 progeny from its mating to a CD-1 dam. **g**, The F_1 pups shown in **f** developed a uniform brown coat, typical of a mating between B6D2F1 (that is, iPS 4N-comp) and CD-1 mice. **h**, A chimaeric mouse (2N) produced from blastocyst injection with IP14D-1 cells. When the mouse is mated to a CD-1 mouse, the progeny are a combination of brown and white coat colours.

embryos were observed at the morula and blastocyst stages, coinciding with the timing of Oct4 expression (Fig. 2e). All the progeny presented with a uniform brown coat colour typical of matings between a black B6D2F1 (that is, iPS 4N-comp) and a white CD-1 mouse (Fig. 2g). This is in contrast to the progeny obtained from the mating of the germline transmittable chimaeric mouse (2N) produced by the same iPS cells by blastocyst injection into a CD-1 mouse (Fig. 2h). This latter process gives rise to a combination of brown- and white-coated offspring. This further demonstrated the viability and normal productivity of the iPS 4N-comp mice, and reflects the true pluripotency of the iPS lines that are capable of producing live iPS 4N-comp mice.

Global gene expression in iPS cell lines that are capable of producing 4N-comp or 2N germline transmittable mice was analysed as reported previously[28] (Fig. 3). The expression of pluripotency markers (*Oct4*, *Nanog* and *Sox2*) as well as other regulators was similar between the iPS and the ES cells, distinct from that of the MEF cells (Fig. 3a and Supplementary Fig. 6). Hierarchical clustering analysis grouped all of the iPS cells with the ES cells, and not with the MEF cells (Fig. 3b). Clearly, the global expression pattern of 4N-comp iPS cells resembles ES cells that are tetraploid competent—an assay considered to be the gold standard for true pluripotency.

We generated 37 iPS lines that demonstrated ES-like characteristics and enhanced developmental potentials through retroviral infection with the four Yamanaka factors. Three iPS lines are capable of generating 4N-comp mice; so far 27 have been born, some of which have matured and are reproductively competent. The reprogramming of these iPS cells should be further investigated to identify

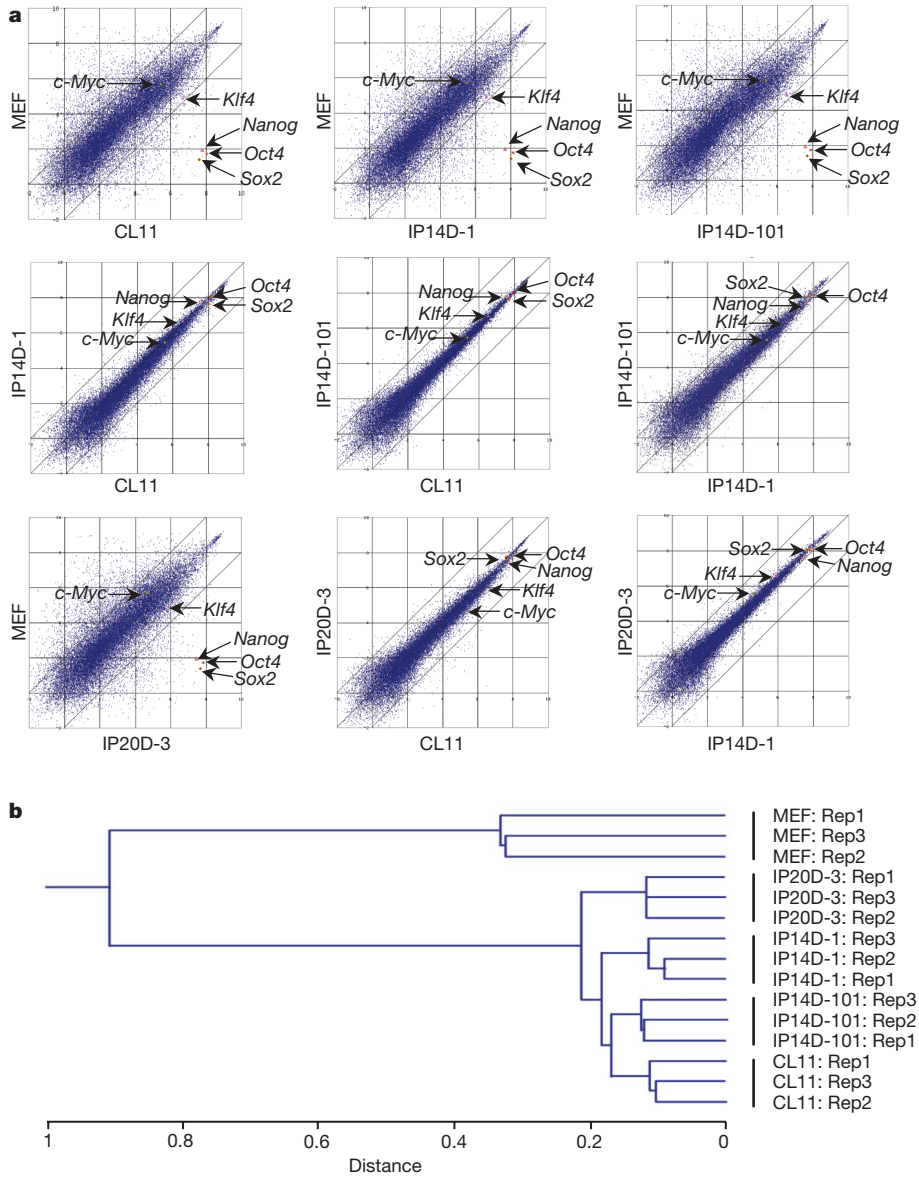

Figure 3 | Global gene expression analysis of iPS cell lines competent for tetraploid complementation. a, Scatter-plots compare the expression values for all probe sets from genome-wide transcription profiling derived from ES (CL11), IP14D-1 and IP14D-101 cell lines that are capable of 4N-comp, or IP20D-3 that is a germline transmittable iPS line, or the parental Oct4–GFP MEF cells that produced IP14D-1 and IP20D-3. Parallel diagonal lines indicate twofold expression difference thresholds. **b**, Hierarchical clustering of microarray data for differentially expressed genes from these various lines. Tree branch distance scale $= 1 - r$; in which r is Pearson's correlation coefficient. Rep, replicate.

factors critical for determining the higher developmental capacity of iPS cell lines derived from clones picked earlier in the reprogramming process, perhaps at time point(s) critical for epigenetic regulation. It would be interesting to determine whether iPS cells produced from direct reprogramming use different mechanisms for altering the genome compared to SCNT. Our result provides the first, to our knowledge, definitive evidence that iPS cells are truly pluripotent, a characteristic that is critical for important applications such as therapeutic interventions.

METHODS SUMMARY

iPS cells were generated from MEF cells with different genetic background as described[1], with the exception of culturing in 20% knockout serum replacement (KOSR) (Gibco) instead of FBS. Thirty-seven colonies were obtained for derivation, and all yielded stable cell lines. iPS cell lines derived at different time points were further confirmed for pluripotent properties by alkaline phosphatase staining and immunofluorescent analysis of SSEA1 (Chemicon), Oct4 (Santa Cruz) and Nanog (Chemicon). Teratoma assays were performed by injecting iPS cells into the subcutaneous flanks of SCID mice, followed by histological examination of the tumours 4–5 weeks later. Bisulphite genomic sequencing, karyotype analysis, SSLP analysis, reverse PCR, and Southern blot analysis were performed. Tetraploid embryo complementation was carried out as described by injecting iPS cells (B6D2F1, black coat origin) into CD-1 tetraploid embryos (white coat colour), and the embryos derived from tetraploid blastocyst injection (4N) were dissected in handling medium on E9.5, E13.5 and the day of birth (E19.5), and analysed for morphology and developmental competency. Finally, global gene expression in ES cells as well as iPS cell lines with different developmental competence was analysed as reported[28].

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

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Author Contributions Q.Z. and F.Z. designed the experiments, supervised lab work, analysed and interpreted data, and wrote the paper; X.Z., W.L., Z.L., L.L., M.T., T.H., J.H., C.G., Q.M. and F.Z. performed experiments; L.L. and W.L. analysed data; W.L. supervised experiments; and X.Z., W.L. and Z.L. contributed to part of the Online Methods section.

Author Information The microarray data in this study have been deposited with the Gene Expression Omnibus repository (www.nbci.nlm.nih.gov/geo) under accession number GSE16925. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to Q.Z. (qzhou@ioz.ac.cn) or F.Z. (fzeng@sjtu.edu.cn).

METHODS

Cell culture. ES cells and iPS cells were cultured as previously described[29] on mitomycin-C-treated MEF cells with DMEM (Gibco) plus 15% FBS (Gibco), 1,000 U ml^{-1} LIF (Chemicon), 2 mM glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 0.1 mM β-mercaptoethanol (Sigma) and 0.1 mM non-essential amino acids. For iPS derivation, DMEM/F12 (1:1, Gibco) and 20% knockout serum (Gibco) was used instead of DMEM with 15% FBS. ES and iPS cells were passaged every 2 days. Oct4–GFP MEF cells (of a B6D2F1 genetic background, that is, F_1 of C57BL/6J × DBA/2J) used for the generation of iPS cells were isolated from E13.5 embryos containing an Oct4–GFP reporter[30], and were cultivated with DMEM plus 10% FBS (Gibco) before retroviral infection. MEF cells for iPS derivation were also made from E13.5 embryos of a C57 × 129S2 background.

Note that all of the animals that were used for cell culture or for reproductive studies were handled according to the Guidelines for the Care and Use of Laboratory Animals established by the Beijing Association for Laboratory Animal Science.

Retroviral production and infection. Retroviral production and infection followed the previously published protocol[25]. In brief, the four retroviral vectors (pMXs-Oct4, Sox2, c-Myc and Klf4) were introduced into plat-E cells using lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's recommendations. After overnight transduction, the medium was replaced. Another 24 h later, the virus-containing supernatants were collected and filtered through a 0.45-μm cellulose acetate filter (Millipore), supplemented with 4 μg ml^{-1} polybrene (Sigma). Oct4–GFP MEF cells (seeded at 2×10^5 cells per 10-cm culture dish) were incubated with virus-containing supernatants for 48 h with medium change at 24 h before replacement with regular media. Two days after infection, the infected Oct4–GFP MEF cells were replated with 2.5×10^4 cells per 35-mm dish on mitomycin-C-treated MEF feeder layers.

Immunofluorescence analysis and alkaline phosphatase staining. Cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.5% Triton X-100 for 30 min followed by blocking with 2% BSA (Sigma). Cells were incubated in primary antibody overnight at 4 °C and secondary antibody at room temperature for 1 h. The following antibodies were used: SSEA1 (Chemicon), Oct4 (Santa Cruz), Nanog (Chemicon). Alkaline phosphatase staining was performed with BCIP/NBT Alkaline Phosphatase Colour Development Kit (Beyotime) according to manufacturer's instructions.

Teratoma formation and histological analysis. iPS cells were trypsinized and suspended at 1×10^7 per ml. One-hundred microlitres of the cell suspension was injected into the subcutaneous flanks of the SCID mice. Four to five weeks later, the mice were euthanized and the tumours were fixed and sliced. Sections were stained with haematoxylin and eosin.

Diploid blastocyst injection and tetraploid embryo complementation. Diploid blastocysts were gently flushed out from the uteri of E3.5 timed-pregnant mice with CZB medium[31]. The generation of mice by tetraploid embryo complementation was carried out as previously described[29]. In brief, two-cell embryos were collected from oviducts of CD-1 females (white coat colour), electrofused to produce one-cell tetraploid embryos that were then cultured in CZB media. Ten to fifteen iPS cells (originally with a B6D2F1 genetic background, or black coat colour) were injected into each tetraploid blastocyst and transferred to CD-1 pseudopregnant recipient females. Embryos derived from tetraploid blastocyst injection (4N) were dissected in handling media on E9.5, E13.5 and the day of birth (E19.5), respectively.

Bisulphite genomic sequencing. Bisulphite treatment of the genomic DNA was performed with the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. *Oct4* and *Nanog* promoter regions were amplified with nested primers (Supplementary Table 2). The first round of PCR was performed as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 59 °C for 45 s, 72 °C for 30 min; and 72 °C for 7 min. The second round of PCR was the same. The PCR products were cloned into pMD18-T vectors (Takara). Ten randomly selected clones were sequenced and analysed.

Karyotype analysis. Karyotype analysis was conducted using standard murine chromosome analysis protocols.

Determination of the SSLP by PCR. Sequences for the primer pairs were found on the Mouse Genome Informatics website (http://www.informatics.jax.org/). DNA was extracted from tail tips of the mouse or cell pellet in culture and typed as described[32]. Products were separated by 3% agarose gels and visualized by ethidium bromide staining.

PCR amplification of lineage-specific microsatellite loci. DNA from mouse tails of CD-1, C57, DBA, B6D2F1, 129, one IP14D-1 4N-comp mouse, one offspring of the IP14D-1 4N-comp mouse, one IP14D-1 chimaera, one IP14D-1 chimaera with germline transfer, one IP14D-101 4N-comp mouse, one IP14D-101 chimaera, and from cell lines of Oct4–GFP MEF cells and IP14D-1 were prepared using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Amplification of lineage-specific microsatellite DNA was performed according to the procedure described previously[33].

Southern blot analysis. To detect the viral integration of the transgene, genomic DNA was extracted by the classical phenol–chloride extraction method. Twenty-five micrograms of genomic DNA was digested with BglII (Takara) for transgenic c-Myc detection and BamH1 (New England Biolabs) for transgene Oct4 detection. Digested DNA was electrophoresed in 0.8% agarose gel overnight and transferred onto positive charged nylon membrane (Millipore). Hybrydization was performed at 67 °C with radioactively labelled cDNAs prepared by Prime-a-Gene Labelling System (Promega). Primers used to synthesize the probes were: *c-Myc* forward, 5′-ACTCCGTACAGCCCTATTTC-3′; *c-Myc* reverse, 5′-TTCA GCTCGTTCCTCCTCT-3′; *Oct4* forward, 5′-TGGACACCTGGCTTCAGA-3′; *Oct4* reverse, 5′-AGCAGTGACGGGAACAGA-3′.

Reverse PCR. Genomic DNA was extracted from the iPS cell lines IP14D-1, IP14D-6 and IP14D-101, as well as from the tails of IP14D-1 4N-comp, IP14D-6 4N-comp, IP14D-101 4N-comp mice and a normal B6D2F1 mouse using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The integration patterns of theses samples were identified by reverse PCR (also known as inverse PCR, iPCR) procedures[34]. Two micrograms of genomic DNA of those mice was digested with two restriction enzymes (BglII and BamHI, New England Biolabs) that have cohesive ends, and cut at least once in pMXs plasmids between the 5′-long terminal repeat (LTR) and the 3′-LTR. The digests were purified by phenol–chloroform extraction and ethanol precipitation, then resuspended in 200 μl ligation reaction with 1,000 U T4 DNA ligase (New England Biolabs). After incubation at 22 °C overnight, ligated DNA was extracted with phenol–chloroform and precipitated with ethanol, then resuspended in 15 μl water. The nested iPCR amplifications across the 3′-LTR junction were conducted by using an Advantage 2.0 PCR kit (Clontech). *Oct4*-specific primers used were: first round: forward, 5′-GCCCCCACTTCACCACACT-3′; reverse, 5′-AGGCCTCGAAGCG ACAGAT-3′; second round: forward, 5′-GAGGTAGACAAGAGAACCTGG AGC-3′; reverse, 5′-GCAAAGTCTCCACGCCAAC-3′.

Reverse transcription and quantitative PCR analysis. To test the expression of pluripotent genes with endogenous and transgenic origin, total RNA was isolated using TRIzol reagent (Invitrogen) and first-strand complementary DNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT (Promega) according to manufacturer's instructions. qPCR was performed using SYBR green (Sigma) on an ABI 7000 instrument. Primer sequences for each gene are listed in Supplementary Table 3.

Microarray analysis. Miroarray analysis was performed as previously reported[28,35]. Total RNA was extracted from three replicates of each cell line, including the following: CL11 (a B6D2F1 background ES cell line from which we recently succeeded in generating live 4N-comp animals, provided by S. Gao, National Institute of Biological Sciences), IP14D-1, IP14D-101 (4N-comp capable iPS cell lines), IP20D-3 (2N germline transmittable) and MEF (Oct4–GFP) cells using TRIzol reagent (Invitrogen). RNA mass and size distribution were determined using the Agilent Bioanalyser with RNA 6000 Nano LabChips, and 2.5 μg of total RNA was used as initial template for biotin-labelled cRNA amplification with GeneChip One-Cycle labelling kit (Affymetrix). Fifteen micrograms per replicate of fragmented cRNA was hybridized to MOE430 2.0 GeneChips (Affymetrix) then washed and stained on fluidics stations and scanned using a GCS3000 scanner according to the manufacturer's instructions. Expression Console (with MAS5) was used to quantify microarray signals with default analysis parameters and global scaling to target mean = 150. Scatter-plot was used to display the expression values for all probe sets from genome-wide transcription profiling derived from the different samples. Hierarchical clustering was performed using the NIA Array Analysis Tool (http://lgsun.grc.nia.nih.gov/ANOVA/index.html) to visualize inter-relationships between different cell lines using all microarray data and the subset of significantly different genes by multi-class analysis of variance (ANOVA) with multiple testing correction. The expression profiles of selected key pluripotent marker genes were also plotted for comparison. The microarray data for each sample were deposited with the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo) under accession number GSE16925.

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