LETTERS

piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells

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Transgenic expression of just four defined transcription factors (c-Myc, Klf4, Oct4 and Sox2) is sufficient to reprogram somatic cells to a pluripotent state[1–4]. The resulting induced pluripotent stem (iPS) cells resemble embryonic stem cells in their properties and potential to differentiate into a spectrum of adult cell types. Current reprogramming strategies involve retroviral[1], lentiviral[5], adenoviral[6] and plasmid[7] transfection to deliver reprogramming factor transgenes. Although the latter two methods are transient and minimize the potential for insertion mutagenesis, they are currently limited by diminished reprogramming efficiencies. *piggyBac* (PB) transposition is host-factor independent, and has recently been demonstrated to be functional in various human and mouse cell lines[8–11]. The PB transposon/transposase system requires only the inverted terminal repeats flanking a transgene and transient expression of the transposase enzyme to catalyse insertion or excision events[12]. Here we demonstrate successful and efficient reprogramming of murine and human embryonic fibroblasts using doxycycline-inducible transcription factors delivered by PB transposition[13]. Stable iPS cells thus generated express characteristic pluripotency markers and succeed in a series of rigorous differentiation assays. By taking advantage of the natural propensity of the PB system for seamless excision[12], we show that the individual PB insertions can be removed from established iPS cell lines, providing an invaluable tool for discovery. In addition, we have demonstrated the traceless removal of reprogramming factors joined with viral 2A sequences[14] delivered by a single transposon from murine iPS lines. We anticipate that the unique properties of this virus-independent simplification of iPS cell production will accelerate this field further towards full exploration of the reprogramming process and future cell-based therapies.

To explore the utility of PB as a vector for somatic cell reprogramming, it was necessary to achieve temporal expression control and transgene silencing, as PB transposons are not purposefully subjected to the same natural silencing process that diminishes retroviral (and less extensively lentiviral) expression[15]. The genes for the four mouse factors (c-*Myc*, *Klf4*, *Oct4* and *Sox2*)[1] were transferred into the PB-TET transposon plasmid (hereafter called PB-TET-mFx; Fig. 1a) under the transcriptional control of the $tetO_2$ tetracycline/doxycycline inducible promoter[16]. All were linked to *βgeo* (a fusion of β-galactosidase and the neomycin resistant gene) through an IRES sequence to allow monitoring the tightness of doxycycline regulation and later demonstration of the reprogrammed cells' capacity for exogenous-factor-independent maintenance. The reverse tetracycline transactivator (rtTA) protein was provided by parental mouse embryonic fibroblasts (rtTA-MEFs) established from 15.5 days post coitum (d.p.c.) ROSA26 rtTA-IRES-GFP knock-in[17] transgenic embryos. rtTA-MEFs were transfected with circular PB-TET-mFx plasmid DNA 'cocktails' in conjunction with a PB transposase expression plasmid[11]. From the time of transfection onwards, fibroblasts were maintained in doxycycline-containing embryonic stem (ES) cell culture conditions without passage. Mouse fibroblasts underwent ES-cell-like colony formation, which resulted in the derivation of self-renewing cell lines displaying key characteristics of reprogramming (Supplementary Fig. 1).

Colonies produced from rtTA-MEFs with PB-TET-mFx cocktails were scored on the basis of morphology, with early foci formation noted on day 6–8 and colonies forming around day 8–10. These were mostly ES-cell-like (Supplementary Fig. 1a), whereas a handful displayed diverse morphologies (data not shown). Adjusting the doxycycline concentration above or below the standard of 1.5 $\mu g\,ml^{-1}$ resulted in subtle declines (150 $ng\,ml^{-1}$) or drastic decreases (15 $ng\,ml^{-1}$ or 15 $\mu g\,ml^{-1}$) in colony numbers (Supplementary Fig. 1b), supporting the notion that factor expression level affects reprogramming rates. In the absence of doxycycline, no reprogramming was noted, nor were any cells found positive for alkaline phosphatase staining (data not shown).

Forty-eight PB-TET-mFx-induced colonies were picked from fibroblast induction fields and passaged on inactivated fibroblast feeder layers. Surviving clones were maintained in doxycycline during establishment, until found to be doxycycline independent in replicate wells. Doxycycline independence occurred as early as day 12 for 3 lines (4D, 4E, 6C), and was achieved by most clones by day 24 (7 out of 39 = 18% on day 15; 21 out of 39 = 54% on day 19; 31 out of 39 = 80% on day 21; 33 out of 39 = 86% on day 23). During establishment, cultures which had not yet achieved autonomous maintenance of reprogramming rapidly flattened and returned to a fibroblast-like state on doxycycline withdrawal (Supplementary Fig. 2a, b) as observed in a prior report[5]. Doxycycline independence was sustained for at least an additional 18 passages, with no apparent disturbance of growth characteristics compared to replica cultures maintained in 1.5 $\mu g\,ml^{-1}$ doxycycline. Doubling time and morphology of PB-TET clones were indistinguishable from those of R1 mouse ES cells[18] (data not shown).

Stable reprogramming by exogenous factor expression results in the sequential activation of ES cell markers[3,5,19]. On day 16 (p2–3, where p indicates passage) the seven doxycycline-independent lines passed the alkaline phosphatase staining criterion (Fig. 1b). These clones tested positive on day 20–22 for the cell-surface marker SSEA1 and nuclear-localized Nanog protein (Fig. 1b). Semi-quantitative reverse transcription polymerase chain reaction (RT–PCR) revealed the expression of ES cell pluripotency markers[20–22], such as Dax1, Eras, Fbxo, Foxd3, Nanog, Rex1 and Zfp296 (Supplementary Fig. 2c). Using 3′ untranslated region (UTR)-specific reverse primers (Supplementary Table 1), the endogenous counterparts of the four mFx transgenes were also shown to be active (Fig. 1c).

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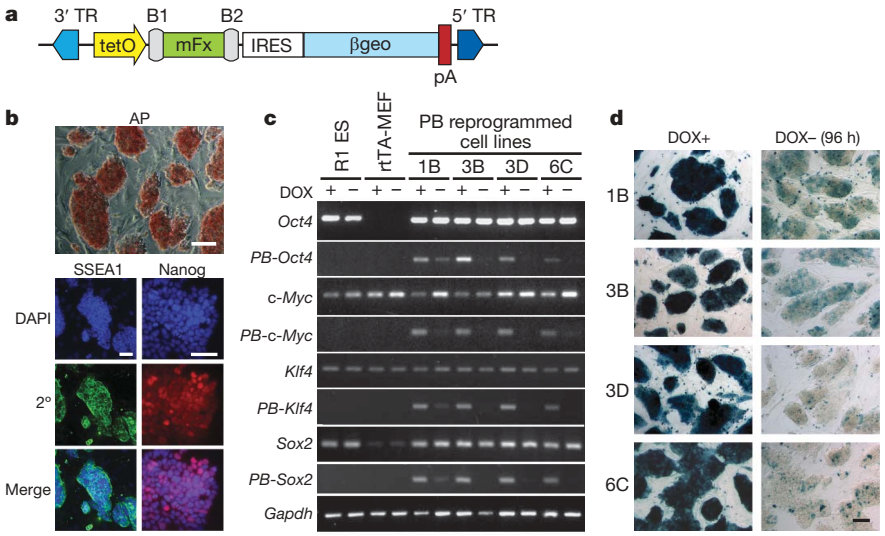

Figure 1 | Cell lines generated by PB-mediated factor transposition are reprogrammed. **a**, The PB-TET transposon vector used to deliver inducible (tetO), reporter-linked (IRES-βgeo-pA) mouse factors (mFx). 3′/5′ TR, PB terminal repeats; B1/B2, post-Gateway cloning sites. **b**, Stable doxycycline-independent cell lines activate alkaline phosphatase (AP), SSEA1 and Nanog. Representative images are from a single cell line (1B). **c**, Doxycycline regulation of PB-delivered factors as monitored by transgene-specific RT–PCR analysis (vector-based reverse primer). Reprogrammed cell lines and controls were grown in the presence (+) or absence (−) of doxycycline for two passages (~96 h). The induction (*Oct4*, *Sox2*) or maintenance (c-*Myc*, *Klf4*) of endogenous gene expression was determined by RT–PCR using 3′-UTR-directed reverse primers. **d**, Residual transgene expression in reprogrammed cell lines visualized as LacZ activity from the transcription-linked *βgeo* reporter gene (Fig. 1a). The level and mosaicism of LacZ after doxycycline withdrawal correlates roughly with basal transcription level detected by RT–PCR. All scale bars are 200 μm.

PB-TET-reprogrammed clones were screened for LacZ expression to determine general transgene activity in the presence and absence of doxycycline (Fig. 1d). RT–PCR, which allows further distinction of individual transgene activity, revealed variable trace expression levels of some transgenes in the uninduced (dox−) state (Fig. 1c). *Oct4* transgene expression could be detected in 1B and to a lesser extent in 3B, and c-*Myc* was maintained minimally in both 1B and 6C. For clones 1B and 3D *Sox2* expression could be detected, whereas *Klf4* appeared silent in all lines except for 1B. Note that RT–PCR detection of c-*Myc*, *Klf4*, *Oct4* and *Sox2* (mFx) transcripts reflects average expression levels for each cell population, and does not expose mosaic expression patterns (as revealed in Fig. 1d).

Genomic Southern blot analysis was used to determine transgene copy number in selected doxycycline-independent mouse PB-TET lines (Supplementary Fig. 3a). The average estimated PB transposon copy number was 9, although co-migrating bands which may obscure the exact total could not be ruled out. Line 3D contains only five transposon insertions. As all four PB-TET-mFx transgenes are represented in 3D (Fig. 1c), three of the four factors are sufficient in single copy for reprogramming.

Through additional transient transposase expression, we exploited the 'cut' step of transposition to remove the transgenes after complete reprogramming. For clone 1B, a broad range was observed in the mobilization rate for each transposon (Supplementary Figs 3b and 4); transposon 8 was excised only once (1 out of 38 = 2.5%) whereas transposon 9 was excised 11 times (11 out of 38 = 29%). Such high variability suggests that the excision frequency is integration-site-dependent. To simplify complete transgene removal we inserted the MKOS sequence (c-*Myc*, *Klf4*, *Oct4* and *Sox2* ORFs linked with 2A peptide sequences[14]) into PB-TET (Fig. 2a). Similar to four-factor reprogramming, induction of rtTA-MEFs with PB-TET-MKOS in the presence of doxycycline yielded colonies after 10–14 days. Out of 48 iPS-cell-like colonies, characterized by pluripotency marker expression and diagnostic Southern analysis (not shown), two single-copy cell lines, designated as scB1 and scC5, were established. Both lines maintained tight regulation of the MKOS reprogramming factors through indirect measurement of the LacZ reporter (Supplementary Fig. 5). Splinkerette PCR[11] determined that PB inserted into the first intron of the *Myo1d* gene (chromosome 11) in scB1, and into an intragenic region of chromosome 16 in scC5 (Fig. 2b). The insertion events were confirmed by locus-specific genomic PCR (Fig. 2d). For both scB1 and scC5, transient PB transposase expression led to the removal of the linked reprogramming factors in >2% of the exposed cells as estimated by the ratio of LacZ-negative sublines in the presence of doxycycline (Supplementary Fig. 5). Sequence analysis across the original insertion sites revealed that in 10 out of 11 subclones the original transposon integration site was reverted to wild type (Fig. 2c). Complete loss of the PB-TET-MKOS transposon was further confirmed by locus- and transgene-specific genomic PCR revealing subclones which were negative for both genome–transposon junction and internal transposon-specific PCRs (Fig. 2d). Even after the exogenous factors were removed, the endogenous pluripotency genes remained actively expressed (Fig. 2e).

Pluripotency of PB-reprogrammed cell lines (1B, 3D, 6C, scB1 and scC5) was demonstrated by their contribution to chimaera development (Fig. 3a and Supplementary Table 2). Embryos were dissected between 10.5 d.p.c. and 15.5 d.p.c. and scored for GFP fluorescence, indicating contribution of reprogrammed ROSA26-rtTA-IRES-GFP parental MEFs. To reveal chimaeric contribution at the cellular level in tissues representative of the three germ layers we treated recipient dams with doxycycline 20 h before dissection, and then LacZ stained and sectioned embryos (Fig. 3b and Supplementary Table 2). Notably, for cell line 1B, many embryos demonstrated high-level chimaeric contribution despite low-level residual factor expression (Fig. 1c, d), suggesting a threshold that permits differentiation of these cells in the embryonic environment. Pluripotency was stringently confirmed by the production of completely iPS-cell-derived 13.5 d.p.c. embryos (Fig. 3c) via tetraploid embryo complementation assay[18], including germ cell formation detected by Vasa expression[23] in the genital ridge (Fig. 3c). Germ cell formation from iPS cells was also detected in genital ridges of standard diploid chimaeric embryos (Supplementary Fig. 6a). Teratomas containing derivatives from all three embryonic germ layers are additional proof that these cells are pluripotent and able to differentiate to complex tissues (Supplementary Fig. 6b). The chimaeric contribution of iPS cells to postnatal animals (Fig. 3d) is a clear testament to PB transposon-assisted complete reprogramming of fibroblasts to iPS cells (PB-iPS) with the capability of building functional adult tissues.

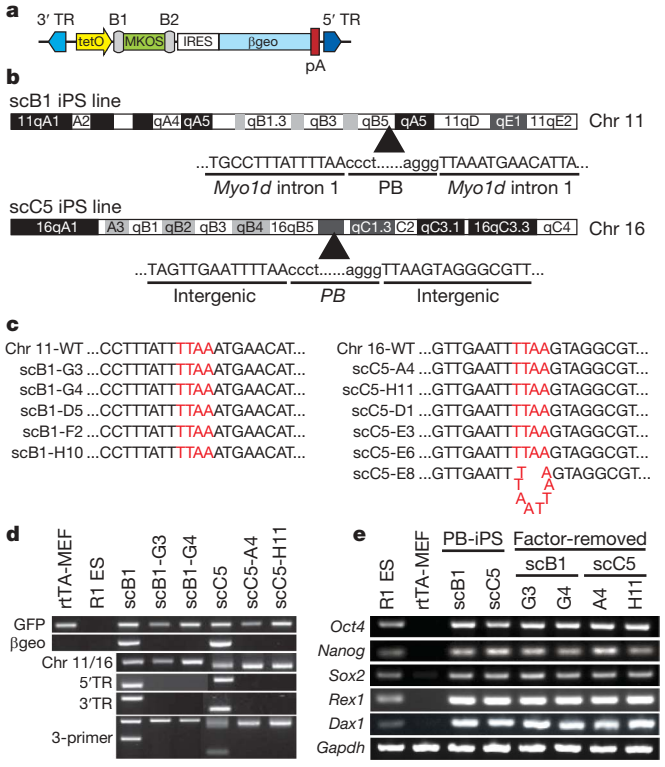

Figure 2 | Seamless factor removal from iPS cells using transposase-stimulated PB excision. **a**, Schematic of the MKOS-containing PB-TET transposon. **b**, Genomic integration site of the individual transposons in scB1 and scC5 lines. Uppercase letters represent flanking genomic sequences whereas lowercase letters are transposon terminal repeat sequences. **c**, Sequence analyses revealed that no mutation was left behind after transposon-mediated removal in most sublines (10 of 11). One single C5 subclone harboured a TTAA duplication at the excision site. **d**, Molecular demonstration of transposon removal in representative subclones. From top to bottom: GFP PCR ensures that all cell lines are derivatives of rtTA-MEFs (R1 ES is a negative control); βgeo PCR detects the presence of transposons regardless of genomic location; Chr-11- and Chr-16-specific PCR across the TTAA tetranucleotide insertion site; 5′ and 3′ junction PCR; 3-primer PCR for the wild-type allele and the transposon–genome junction. scB1 and scC5 are hemizygous for the transposon and amplify the wild-type allele. **e**, RT–PCR analysis of the single-transposon-induced iPS cell lines and their factor-removed derivatives reveals maintenance of characteristic pluripotency gene expression. R1 ES cells and parental rtTA-MEFs serve as positive and negative controls, respectively.

We successfully used a PB-CAG-rtTA transposon together with PB-TET-mFx for doxycycline-regulated reprogramming of normal human embryonic fibroblasts. Colonies induced via transposition were picked between day 14 and day 28. Four out of five alkaline-phosphatase-positive clones became doxycycline independent 1 week after isolation. Factor expression in the uninduced state was not detectable by LacZ staining (Supplementary Fig. 7a) and only trace amounts of transcription were detected in colonies by semi-quantitative RT–PCR (Supplementary Fig. 7b). Expression of the endogenous forms of the inducer genes, however, reached levels characteristic of human ES cells (Supplementary Fig. 7b) along with characteristic pluripotency marker genes (Supplementary Fig. 7c) including nuclear-localized NANOG (Supplementary Fig. 8). Spontaneous differentiation after bFGF withdrawal from the medium gave rise to cystic embryoid bodies and to various differentiated cell types, including AFP (endoderm), αSMA and vWF (mesoderm), or βIII tubulin and GFAP (ectoderm) positive cells (Supplementary Fig. 9). In accordance to an earlier report[24], our observations indicate that mouse factors effectively reprogram human cells, underlining the functional conservation of these transcription factors between the two species.

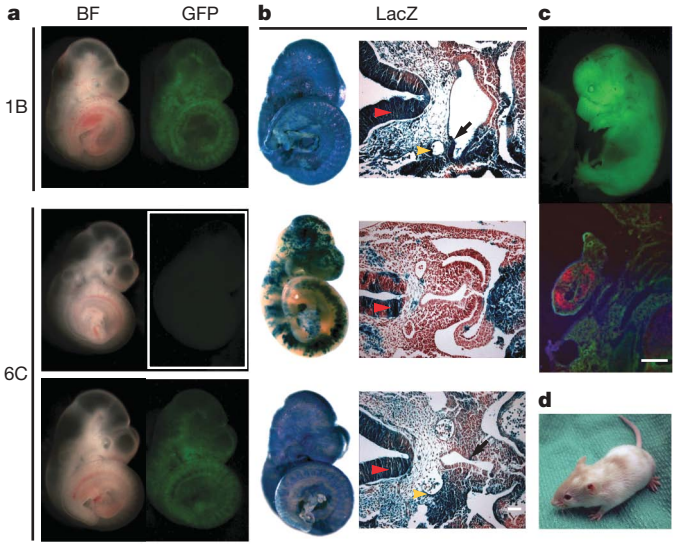

Figure 3 | Cell lines reprogrammed by PB-mediated factor transposition are pluripotent. **a**, Contribution of PB-TET cell lines to embryonic development. Chimaeras dissected at 10.5 d.p.c. with the strongest contribution of PB-iPS-derived cells were easily detected as GFP positive (BF, bright field). **b**, Whole-mount LacZ staining of chimaeric embryos and their sections show contribution of PB-iPS LacZ-positive cells to derivatives of all three embryonic germ layers. Red arrowhead, neural tube (ectoderm); yellow arrowhead, dorsal aorta (mesoderm); black arrow, foregut epithelium (endoderm). **c**, Completely iPS-cell-derived (1B) 13.5 d.p.c. embryo generated by tetraploid embryo complementation. Immunohistochemistry on sections through its genital ridge shows the iPS cell contribution to germ cells (Vasa+, red). **d**, Adult chimaera obtained by aggregating 1B PB-iPS cells with diploid eight-cell-stage ICR (albino) embryos. All scale bars are 100 μm.

Diploid chimaeras from three mouse iPS cell lines (1B, 6C and 3D) were used to derive secondary MEFs, tracking the iPS-cell-derived component (secondary fibroblast (2°F)/1B, 2°F/6C and 2°F/3D) by GFP expression (Figs 3a and 4). Doxycycline treatment had a marked effect on 2°F/1B and 2°F/6C, which initiated early signs of reprogramming within 2 days, including cellular aggregation (Supplementary Movie). Proliferation also increased markedly, (Fig. 4 and Supplementary Fig. 10a) as initial contribution (10–18% on day 0) reached 90% by day 10. These physical changes were reflected at the molecular level by the activation of alkaline phosphatase (not shown) and SSEA1. The latter showed very rapid activation—detected as early as day 2, expressed by nearly 50% of 2°F/1B and 2°F/6C cells on day 4 and greater than 80% on day 8 (Fig. 4 and Supplementary Fig. 10b). Nanog activation occurred four days earlier (by day 9) in 2°F/1B than in 2°F/6C (Supplementary Fig. S11), highlighting clonal variation and flexibility in the reprogramming process. Overall, PB-iPS-cell-derived fibroblasts displayed a much more rapid initial response to reprogramming factor induction than that previously reported for the doxycycline-inducible lentivirus system[25], which may reflect a high level of instability for lentivirus-inserted transgene expression. Single-cell sorting of 2°F/6C cells into 96-well plates revealed that 56 of 192 single cells (29%) were capable of forming colonies. Thirty-nine out of forty-four (89%) established single-cell clones evaluated for Nanog expression were found positive on day 13 of induction. In contrast, line 2°F/3D was inefficient at secondary iPS cell production, as few doxycycline-induced colonies formed and expression of alkaline phosphatase and SSEA1 was delayed (not shown). The PB-TET-Oct4 transgene was noticeably less active in 2°F/3D as compared to 2°F/1B and 2°F/6C (Supplementary Fig. 10c), signifying the need for a sufficient level of Oct4 expression even in secondary iPS cell induction.

Successful transposon-based reprogramming of fibroblasts to iPS cells represents a significant advance in current methods of transgene delivery. First, PB transposition permits technical simplification and

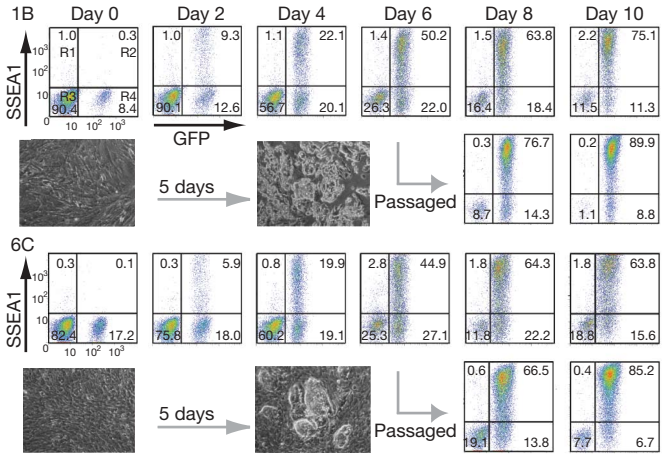

Figure 4 | Properties of secondary fibroblast reprogramming. Fluorescence-activated cell sorting (FACS) analysis establishing the dynamics of SSEA1 activation in 2°F/1B and 2°F/6C cell lines. The inset shows colony formation as early as day 5. On day 6 of reprogramming gene induction, the cultures were also passed by standard trypsinization and analysed in parallel to eliminate the negative effect of cell overgrowth.

improved accessibility of reprogramming methodology by making use of effortless plasmid DNA preparation and commercial transfection products for delivery. This eliminates the need for specialized biohazard containment facilities or the production of high-titre, limited-lifetime viral stocks[26]. Second, the range of somatic cell types that could be used for reprogramming is not limited by a decreased susceptibility to viral infection[26]. Third, PB-mediated delivery will allow the option of xeno-free production of human iPS cells contrary to current viral production protocols that use xenobiotic conditions. Finally, accurate transgene removal through transposase expression has been demonstrated in various cell lines[8,11,12,27–29]. We have harnessed this potential and show here that the reprogramming factors can be removed without a trace from iPS cells once exogenous expression becomes dispensable. These four key characteristics of PB transgenesis mark important advances towards achieving clinically acceptable methods of deriving reprogrammed cells. Complementary, secondary fibroblasts or alternative secondary cell types with a combinatorial removal of proven reprogramming transgene insertions may provide an enormously powerful tool for high-throughput screening to explore further the mechanisms that have roles in the reprogramming process.

METHODS SUMMARY

PB expression vectors were generated using standard cloning procedures, whereas reprogramming factors were shuttled from retroviral sources using Gateway cloning (Invitrogen). Plasmid DNA for fibroblast transfection was prepared using a QIAprep Spin Miniprep Kit (Qiagen).

MEFs were isolated from 15.5 d.p.c. ROSA26 knock-in rtTA-IRES-GFP[17] embryos. HEFs were derived from 12-week abortuses. Fibroblasts were seeded in ES media at 1.25×10^5 cells per 10 cm^2 (MEFs) or 6.25×10^4 cells per 10 cm^2 (HEFs) and transfected with PB-mFx transposon vectors and PB transposase expression plasmid 24 h later. For PB-TET-mFx transfections, expression was induced with 1.5 $\mu g\,ml^{-1}$ doxycycline the following day. Forty-eight hours after transfection, cells were fed with fresh ES media daily without passage. The resulting colonies were picked on day 10–12. iPS clones were maintained on inactivated feeders, or gelatin-coated dishes.

LacZ staining was performed overnight on 0.25% glutaraldehyde-fixed cells. Alkaline phosphatase staining was done according to the manufacturer's specifications (Vector Labs). RT–PCR used cDNA prepared with the QuantiTect Reverse Transcription Kit (Qiagen). Southern blotting was carried out using standard methods and DIG-labelled probes for immunodetection (Roche). Immunostaining was performed on cells grown and fixed on chambered slides (Nunc) or 4-well dishes. Flow cytometry used live cells stained with SSEA1.

Mouse PB-TET clones were grown in the absence of doxycycline for at least three passages before aggregation with diploid or tetraploid Hsd:ICR(CD-1) or diploid C57BL/6 host embryos[30]. The prenatal embryos were dissected, scored for GFP and then further processed to perform immunohistochemistry or to derive secondary MEFs. Embryos prepared for LacZ staining and sectioning were treated with doxycycline *in utero* 20 h before dissection. Teratoma formation using PB-TET clones was performed as previously described[1]. Differentiation of human iPS was spontaneous on Matrigel following plating of embryoid bodies formed in AggreWell (SCI) dishes.

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

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

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Author Contributions K.W. designed the experiments, cloned the transposon vectors, isolated and transfected fibroblasts, cultured mouse PB-iPS lines, performed alkaline phosphatase, LacZ and immunostaining, FACS analysis, dissected embryos, prepared DNA and performed Southern blotting, collected, analysed and interpreted data, and wrote the manuscript. I.P.M. designed experiments and assisted with initial cloning. P.M. and R.D. isolated fibroblasts, and assisted with cell culture, immunostaining and embryo dissections. M.M. transfected human fibroblasts, cultured human PB-iPS lines, performed alkaline phosphatase staining, immunostaining and differentiation assays. R.H. and K.W. performed the single transposon reprogramming studies and the removal of factors from iPS cells. R.C. carried out RT–PCR reactions. W.W. and P.L. provided the *PB-PGK-neo-bpA* and pCyL43 transposase plasmids, and guidelines for their use. M.G. generated and coordinated the iPSC chimaera production. K.K. performed immunostaining on induced secondary fibroblasts. H.-K.S. and I.P.M. performed teratoma assays and collected microscopy images. A.N. was responsible for the project concept, supervised the experiment design and data interpretation, and wrote the manuscript. All authors were involved in initial project design, discussed the results and contributed to the manuscript preparation.

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METHODS

Plasmid vector construction. PB-TET was constructed by exchanging the 5′/3′-TR-flanked *PGK-neo* cassette of *PB-PGK-neo-bpA*[11] with a blunt NotI/HindIII fragment containing $tetO_2$ (provided by S. Mohammadi) via digestion with NheI and HindIII (both polished), resulting in PB-$tetO_2$. The NheI and SalI (polished) IRES-βgeo-bpA element from pIFS (provided by J. Dixon) was ligated into PB-$tetO_2$ prepared by NheI and SmaI digestion to yield PB-$tetO_2$-IRES-βgeo. Finally, a Gateway RfA cassette (Invitrogen) maintained in the polished NotI site of pBluescriptKS+ (Stratagene) was inserted by digestion with SacII and SpeI into PB-$tetO_2$-IRES-βgeo digested with SacII and NheI. For PB-CAG, the *PGK-neo* cassette was removed by digestion with NheI (polished) and HindIII and replaced with a CBA-RfA-rβgpA cassette released by OliI and HindIII digestion from a constitutive gene expression construct (I.P.M., K.W. and A.N., unpublished data). The four reprogramming factors were Gateway cloned from retroviral backbones (Addgene) into the PB-TET transposon vector using pDONR221 (Invitrogen) as an intermediate. The Tet transactivator was amplified from pTet-On Advanced (Clontech) using *attB1/2* primers (Supplementary Table 2) and similarly Gateway cloned into PB-CAG. The MKOS element from pCAG2LMKOSimO (ref. 14) was cloned into pENTR2B using EcoRI before Gateway shuttling. The Tet transactivator was amplified from pTet-On Advanced (Clontech) using *attB1/2* primers (Supplementary Table 1).

Fibroblast isolation. 15.5 d.p.c. ROSA26-rtTA-IRES-GFP embryos (from *Gt(ROSA)26Sor*$^{tm1.1(rtTA,EGFP)Nagy}$) were decapitated, eviscerated, dissociated with 0.25% trypsin, 0.1% EDTA and plated in DMEM, 10% FBS, penicillin–streptomycin and glutamax. HEFs were isolated from 12-week-old abortuses and maintained in DMEM, 15% human serum, 10 ng ml^{-1} bFGF, penicillin–streptomycin, glutamax, β-mercaptoethanol and NEAA.

PB transfection and cell culture. MEFs were seeded in DMEM, 15% FBS, penicillin–streptomycin, glutamax, β-mercaptoethanol, sodium-pyruvate, non-essential amino acids and LIF on gelatinized (0.1%) 6-well dishes at a density of 1.25×10^5 cells per 10 cm^2. After 24 h culture, FugeneHD (Roche) was used to transfect cells with 10 ng, 100 ng, or 400 ng of each mFx transposon (25 ng, 50 ng, or 100 ng for PB-TET-MKOS) plus 100 ng of pCyL43 PB transposase plasmid[11] (normalized to 2 μg total DNA with empty pBluescriptKS+) at a Fugene:DNA ratio of 8 μl:2 μg. After 24 h, the media was supplemented with doxycycline (day 0), and changed entirely 48 h after transfection. Cells were fed daily with doxycycline-containing media (1.5 μg ml^{-1}, unless otherwise indicated). Colonies were picked in 96-well format over days 10–14 and cultivated on mitomycin-c-arrested MEFs. For PB-TET induced clones, doxycycline treatment was maintained until day 16–24. iPS cells for DNA or RNA preparation were grown on gelatin. Established iPS cells were passaged 1:6 every 48 h. Transfection of HEFs was performed similarly, except fibroblasts were initially seeded in DMEM supplemented with 15% human serum, 10 ng ml^{-1} bFGF, penicillin–streptomycin, glutamax and non-essential amino acids at a density of 6.25×10^4 cells per 10 cm^2, and grown in HEScGRO (Millipore) 48 h after transfection. Doxycycline (1.5 μg ml^{-1}) was added 24 h after transfection and withdrawn a week after picking. Colonies were initially passaged mechanically 1:2, and later with TripLE Select (Invitrogen) 1:4 every 7 days. Human iPS cells were maintained on inactivated MEFs in KO-DMEM, 20% serum replacement, 10 ng ml^{-1} bFGF, penicillin–streptomycin, glutamax and non-essential amino acids.

Southern blotting. Ten micrograms of genomic DNA from R1 ES cells, PB-iPS lines or rtTA-MEFs was digested with BamHI overnight, resolved by gel electrophoresis, and transferred to Hybond N+ (GE Healthcare). A *neo* probe PCR fragment prepared with DIG High Prime DNA Labelling and Detection Kit II (Roche) was used to detect transposon insertions (~25 ng probe per ml hybridization solution).

Splinkerette, genomic and RT–PCR. Splinkerette PCR to determine PB genomic integration sites was performed as described[11]. TA-cloned PCR products were sequenced bidirectionally with M13 forward and reverse primers. PB insertion loci were determined using BLAST. Genomic PCR on factor-removed PB-iPS lines was performed using primers described in Supplementary Table 1. Approximately 100 ng of genomic template DNA was amplified using Qiagen Taq (Qiagen) with the inclusion of Q-Solution. Highly repetitive sequences on chromosome 16 required nested PCR. Three-primer PCR amplification used PB-3F in conjunction with the chromosome-specific primer set. Standard PCR conditions were: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s; ×35 cycles. RNA was collected using the RNeasy Mini Kit (Qiagen), quantified and treated with gDNA WipeOut and cDNA prepared with the QuantiTect Reverse Transcription Kit (Qiagen). For each RT–PCR reaction, we used 50–100 ng of cDNA and primers listed in Supplementary Table 1. Standard PCR conditions were: 94 °C for 30 s, 58–62 °C for 30 s, 72 °C for 15–30 s; ×30–35 cycles.

PB transgene removal. Stable PB-TET clones were transiently transfected with pCyL43 transposase (2 μg DNA:8 μl FugeneHD). After puromycin (1 μg ml^{-1}) treatment for 3 days to eliminate non-transfected cells, the cells were plated at clonal density (~500–1,000 cells per 60 cm^2) on inactivated MEFs. Clones were picked after 5 days further culture.

LacZ/alkaline phosphatase staining. Cells and embryos were fixed with 0.25% glutaraldehyde, rinsed in wash buffer (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet-P40 in PBS) and stained overnight (~16 h) in LacZ staining solution: 20 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mg ml^{-1} X-gal in PBS. Embryos were embedded in paraffin, sectioned and counterstained with neutral red. Staining for alkaline phosphatase activity was performed on cells without fixation using the Vector Red Kit (Vector Labs).

Immunostaining and flow cytometry. Cells were grown on inactivated MEFs on gelatin-coated Lab-Tek borosilicate glass slides (Nunc) or 4-well dishes containing gelatinized or Matrigel-treated glass coverslip inserts. Samples were washed with PBS, fixed in 4% PFA/PBS for 10 min at 25 °C, permeabilized with 0.3% Triton X-100 in PBS for 10 min at 25 °C, blocked in 5% goat serum for 1 h, and primary antibody was added overnight at 4 °C (mNanog, AB5731, Chemicon, 1:1,000; hNanog, 0002P-F, ReproCell, 1:200; SSEA1, SSEA4, Tra1-60, Tra1-81, 1:5, provided by P. Andrews; muscle actin, M0635, DakoCytomation, 1:100; βIII-tubulin, TU-20, Millipore, 1:100; HNF-3β, sc-9187, Santa Cruz, 1:100; GFAP, Z0334, DakoCytomation, 1:200; AFP, MAB1369, R&D Systems, 1:200; vWF, A 0082, DakoCytomation, 1:200). Samples were washed in PBS and secondary antibody (cy3 IgG, 1:1,000; Alexa488 IgG or IgM, 1:400; Alexa594 IgG, 1:200) was added for 1 h at 25 °C. After washing, samples were mounted in Vectashield with DAPI (Vector Labs). Genital ridges were pre-fixed with 4% paraformaldehyde in PBS for 1 h at 25 °C, embedded and cryosectioned at 30 μm thickness, washed, blocked and incubated overnight at 4 °C with rabbit anti-primordial germ cell marker (DDX4/MVH, ab13840, abcam, 1:400). Sections were washed in PBS and secondary antibody (cy3 IgG, 1:500) was added for 2 h at 25 °C. Immunofluorescence was visualized and acquired using a Zeiss LSM 510 confocal microscope. Doxycycline-induced fibroblast pools from PB-iPS chimaeric mice were diluted to ~15% GFP positive 2°F/1B and 2°F/6C representation with wild-type ICR MEFs before seeding. Cells were washed once in PBS containing 5% FBS, incubated with anti-SSEA1 antibody (1:200) for 30 min on ice, washed twice in PBS:5% FBS, incubated with Alexa647-conjugated secondary antibody for 30 min on ice, washed twice and resuspended in PBS:5% FBS for analysis on a FACS-Calibur. Single GFP-positive cell plating from PB-iPS chimaeric MEF pools was done with a FACS-Aria.

***In vitro* differentiation assays.** Human PB-iPS lines were dissociated and used to generate embryoid bodies by aggregation in AggreWell 400 plates (StemCell Technologies) in 15% FBS DMEM with all additives except LIF and bFGF, in the absence of doxycycline. After 14 days growth, embryoid bodies were plated on Matrigel-coated coverslips or 4 chamber slides. Immunohistochemistry was performed after an additional 10 days culture.

Teratoma formation. Cell lines were suspended in DMEM containing 10% FBS, and 100 μl (1×10^6 cells) injected subcutaneously into both dorsal flanks of nude mice (CByJ.Cg-Foxn1nu/J) anaesthetized with isoflurane. Six weeks after injection, teratomas were dissected, fixed overnight in 10% buffered formalin phosphate and embedded in paraffin. Sections were stained with haematoxylin and eosin.

Generation of chimaeras. Doxycycline-independent PB-TET iPS clones were collected as cell clumps of ~8–15 cells from gelatinized dishes by gentle trypsinization. For diploid chimaeras, 2.5 d.p.c. Hsd:ICR(CD-1) or C57BL/6 embryos were aggregated with PB-iPS cell clumps, and cultured overnight at 37 °C in 5% CO_2 in KSOM medium (Millipore). For tetraploid embryo complementation, two-cell-stage Hsd:ICR(CD-1) embryos (1.5 d.p.c.) were electrofused with a CF-150B Pulse Generator (BLS) and cultured overnight, and aggregated as described above. All embryos were transferred into pseudopregnant recipient ICR females 24 h later[30]. Dissected embryos were examined for GFP using a Leica MZ16 FA stereomicroscope, or left to term to verify coat colour contribution. For LacZ detection, pregnant dams were treated with doxycycline (1.5 μg ml^{-1} doxycycline; 5% sucrose in water) 20 h before dissection.