Efficient *p53* gene targeting by homologous recombination in rat-induced pluripotent stem cells

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Abstract

Objectives: To generate rat induced pluripotent stem cells (iPSCs) using the PiggyBac (PB) transposon system, and to explore whether these iPSCs would be amenable to genetic manipulation.

Materials and methods: The PB transposon system was used to reprogramme rat embryonic fibroblasts (EF) to become iPSCs. Cells were identified with regard to pluripotency and differentiation capacity *in vitro* and *in vivo*, population growth characteristics and gene expression; furthermore, targeting vector was electroporated into them. Correct recombination colonies were acquired by positive and negative selection, and then phenotype confirmed by Southern blotting.

Results: The rat EF cells were reprogrammed into iPSCs successfully, using the PB transposon system. Cell morphology was found to display characteristics of rat embryonic stem cells (ESCs) and results of immunofluorescence staining and PCR indicated that they expressed pluripotency markers. *In vivo* and *in vitro* differentiation experiments proved that the cells could differentiate into all phenotypes from three germ layers, and to form chimaeras with high rat iPSC contribution. After electroporation with *p53* targeting vector, approximately $(5.44 \pm 0.74) \times 10^{-6}$ colonies tolerated selection. Southern blotting confirmed that *p53* gene was targeted successfully in the colonies.

Conclusion: The PB transposon system proved to be an effective method for reprogramming of rat EF cells into iPSCs. The rat iPSCs were amenable

to gene targeting mediated by routine homologous recombination.

Introduction

In 2006, Yamanaka *et al.* reported generation of iPSCs from mouse somatic cells. Like mouse ESCs, mouse iPSCs expressed alkaline phosphatase (AKP), generated chimaeric mice, and contributed to germ-line transmission (1, 2). Subsequent studies demonstrated that human (3–5), mouse (1, 2), rat (6–8) and rabbit (9) iPSCs could be generated using the same, or modestly amended, sets of reprogramming factors, offering the possibility of generating disease model iPSCs to explore genetic determinants of such disease. Mouse and human iPSCs have been demonstrated to be powerful tools for *in vivo* and *in vitro* study of stem-cell biology, cell differentiation, tissue engineering, new therapeutic approaches, and as potential substrates for genetic manipulation (10). Moreover, ESC-like properties of iPSCs allowed for investigation of early embryonic developmental phenomena in a culture system. However, relative to studies of mouse and human iPSCs, studies of rat iPSCs have been lacking. To accelerate discovery of genetic determinants of diseases in this important rat strain, we set out to generate disease model-competent iPSCs.

In 2008, germ-line-competent rat ESCs were established by use of two or three kinase inhibitors, including glycogen synthase kinase 3 (GSK3) inhibitor, mitogen-activated protein kinase (MEK) inhibitor and fibroblast growth factor receptor tyrosine kinase inhibitor, in the culture medium (11, 12). Additionally, rat iPSCs (6, 7) were successfully established by retroviral or lentiviral introduction of transcription factors. Of these, some rat iPSCs displayed germ-line competency (7). Recently, transgenic (13) and knockout rats have been successfully generated by targeted gene manipulation in rat ESCs. (14), strongly indicating that rat iPS with same characteristics of rat ESCs can be used in gene targeting. However, to date, no FHH rat iPSCs have been reported to

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be generated using a no-virus system. No evidence has been shown that adult cell-derived rat iPSCs could be amenable to genetic manipulation.

In the study described here, we successfully generated iPSCs rat EF cells using the PB transposon system combined with application of 2 inhibitors. The rat iPSCs displayed ES cells with typical mouse- and rat-morphology and self-renewal potential *in vitro* over long periods of time (more than 40 passages). In suspension culture, they formed embryonic bodies (EBs) containing various cell types as those originating from all three germ layers. Most importantly, these iPSCs contributed to chimeras in a major way. To ensure whether the iPSCs would be amenable to genetic manipulation, *p53* gene was successfully targeted in these established rat lines. Cell lines and information obtained in this study will accelerate generation of rat iPSCs and guide stem cell biologists in appropriate genetic manipulation of iPSCs from rat.

Materials and methods

Culture of embryonic fibroblasts

Rat and mouse embryonic fibroblasts (EF) were prepared from e14.5 embryos cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal beef serum (FBS) (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 1% non-essential amino acid and 0.1 mM 2-mercaptoethanol. Rat iPSCs were maintained on mitomycin-c treated DR4 mouse EF cells in N2B27 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1000 U/ml of rat LIF, 3 mM of GSK3 inhibitor CHIR99021 (Axon Medchem BV, Groningen, the Netherlands), and 1 mM MEK inhibitor PD0325901 (Stemgent, Cambridge, MA, USA), with or without 2 mM FGF receptor inhibitor SU5402 (Calbiochem, La Jolla, CA, USA). Rat iPSCs were trypsinized to single cells using 0.05% trypsin-EDTA (Sigma, St. Louis, MO, USA) and plated into new wells with mouse EF (MEF) feeder every 3 or 4 days.

Reprogramming rat EF cells using transposon vectors

Rat EF cells were plated into 6-well plates (5×10^5 cells/well) one day before transfection. The following day (day 0), 2.0 µg pCMV-mPBBase and various quantities of plasmid harboring the PB transposon, were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. On day 1, transfected rat EF (REF) were digested and replated on to DR4 feeder layers at split ratio of 1:20, in MEF medium; on day 2, mouse ES medium was applied

and 2 mM valproic acid (VPA) was added to culture medium between days 2 to 7. Medium was refreshed every other day. On day 7, medium was changed to N2B27 with either 2 or 3 inhibitors. Medium was refreshed every other day. On day 10, colonies were either stained using an alkaline phosphatase detection kit (Chemicon, Temecula, CA, USA) and counted, or picked and further expanded.

Immunofluorescence staining of rat iPS colonies

Cells were fixed using 4% paraformaldehyde/PBS for 15 min, then were permeabilized by 0.05% Triton- $\times$ 100/PBS for 10 min at room temperature. They were then blocked in 1% BSA/PBS for 1 h at room temperature. Cells were washed in PBS and incubated with anti-NANOG antibody (Abcam, Cambridge, MA, USA), anti-OCT4 antibody (Santa Cruz, CA, USA) and anti-SOX2 antibody (Santa Cruz, CA, USA) overnight at 4 °C. After being washed three times in PBS, cells were labelled with Alexa 488 or 555-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature and were then washed in PBS (3×10 min); nuclei were counterstained with 0.5 µg DAPI in PBS for half an hour at room temperature. After final washing in PBS (3×10 min), specimens were analysed using a fluorescence microscope.

RT-PCR analysis

Total RNA prepared using RNAeasy kit (Qiagen, Hilden, Germany) was used as a template for reverse transcription-polymerase chain reaction III (RT-PCR). PCR amplification for different genes was performed using GoTaq[®] Polymerase (Promega, Madison, USA). PCR products were visualized using ethidium bromide on a 1.2% agarose gel. Primer sequences are listed (Table 1).

Teratoma investigation

Suspensions of 5×10^6 single cells were injected intratestis, into each testis, of 6 to 10 week-old non-obese diabetic/severe combined immune deficient (NOD/SCID) mice. After 4–8 weeks, tumours were harvested and processed for haematoxylin and eosin staining. After being embedded in paraffin wax, tumours were sectioned and standard histopathological analysis was performed, following haematoxylin and eosin (H&E) staining.

Blastocyst injection

Rat blastocysts, E4.5 day post-coitum (d. p. c), were collected by noon on the day of injection and cultured

Table 1. Primers of RT-PCR

Gene	Forward (5'–3')	Reverse (5'–3')
<i>Oct4</i>	GGGATGGCATACTGTGGAC	CTTCCTCCACCCACTTCTC
<i>R-Nanog</i>	GCCCTGAGAAGAAAGAAGAG	CGTACTGCCCCATACTGGAA
<i>M-Nanog</i>	ATGAAGTGCAAGCGGTGGCAGAAA	CCTGGTGGAGTCACAGAGTAGTTC
<i>Sox2</i>	GGCGGCAACCAGAAGAACAG	GTTGCTCCAGCCGTTTCATGTG

for 2–3 h in M16 medium, to ensure cavitation; well expanded blastocysts were used for microinjection. iPSCs were disaggregated in accutase and injected into F1 hybrid blastocysts from crosses between SD (Charles River) and FHH rats. Ten to twelve injected blastocysts were then transferred into the uterine horn of each E 0.5 pseudopregnant female SD rats. Pups were collected to identify contribution of rat iPSCs to somatic tissue cells, by coat colour and GFP fluorescence.

Construction of *p53* gene targeting vector

We had selected the FHH strain, (source of genomic DNA for the homology arms in the target vector), for gene targeting experiments. An FHH BAC library was screened by primers and a *p53* BAC clone was isolated. A 4.7 kb fragment spanning this region was amplified from *p53* BAC clone by PCR, using oligonucleotide primers based on genomic sequence information available for the Brown Norway (BN) strain. Subfragments of this amplicon, flanking exon 3, provided 5' and 3' homology arms used to encompass a dual positive/negative selection cassette within the *p53* gene targeting vector. This cassette contains a PGK-neo transcription unit to allow positive selection of G418-resistant transfectant, and a thymidine kinase (*TK*) mini gene - inserted in the 3' homologous arm, that enables negative selection.

Electroporation

The targeting vector containing the neo/tk double selection cassette was linearized with Hind III. Rat iPSCs were electroporated in PBS containing 50 mg linearized *p53* targeting vector using the Bio-Rad GenePulser apparatus (0.8 kV, 2.5 mF). Electroporated cells were plated into 10 cm² wells containing 2i medium and feeder cells. G418 (350 mg/ml) was added 48 h post-electroporation and number of G418-resistant colonies was counted 9–10 days after electroporation. FIAU (5 mM) was applied at either day 9 or following picking and replica-plating of individual G418-resistant clones.

Southern blotting analysis

Genomic DNA (10 µg) was digested overnight with 30 unit restriction enzyme in a volume of 50 µL, then redigested with 10 unit enzyme for 2–3 h; samples were loaded on 0.7% agarose gels and run at 60 V overnight. Gels were then denatured for 1 h in 1.5 M NaCl and 0.5 M NaOH, neutralized for 1 h in 0.1 M Tris-HCl pH 7.5 and 0.5 M NaCl, washed in 2 × SSC, and blotted for 48 h with 20 × SSC on Zeta-Probe GT genomic tested blotting membranes (Bio-Rad). Membranes were then washed in 2 × SSC, UV-cross-linked, and stored at –20 °C. For hybridization, membranes were preincubated in buffer (1% BSA, 1 mM EDTA, 0.5 M phosphate buffer, 0.7% SDS) for 1 h at 65 °C, with rotation. DNA fragments used as hybridization probes were denatured and labelled using a Dig DNA Labeling kit (Roche, USA). After being denatured, labelled probes were added to hybridization buffer and membranes were rotated overnight at 65 °C. Washing buffer (2 × SSC, 0.5% SDS) was pre-warmed to 65 °C, and membranes were washed three times (5, 30 and 15 min) at 65 °C, with shaking. The hybridization signal was detected using Dig Luminescent Detection Kit (Roche). Photographs of all Southern blots were scanned and segments were excised using Adobe Photoshop software.

Results

Generation of iPSCs from rat EF cells by the PiggyBac transposon

The rat iPSCs had been reprogrammed from rat EF cells using PB transposon-based reprogramming vectors (15, 16), which contained open reading frames of *Oct4*, *Sox2*, *Klf4* and *Myc* (OSKM) or *Oct4*, *Sox2*, *Klf4*, *Myc* and *Lin28* (OSKML) combined into a single open reading frame, separated by sequences encoding 2A peptides and placed under the constitutively active CAG promoter. The time schedule for reprogramming of the rat iPSCs is summarized in Fig. 1a. A quantity of 2 µg of PB transposon and a quantity of 2 µg of PB transposase expression vector had been co-introduced into FHH EF

cells using lipofection (Fig. 1b). One day after transfection, we had replated transfected EF cells on to a feeder layer at split ratio of 1:20 then grew them in MEF medium. On day 2, medium had been changed to mouse ES medium. After 4 days culture, medium had been changed to N2B27. On day 7, we had added 2 or 3 inhibitors to the medium. Under these conditions, colonies became visible around day 5 without VPA and day 7 with VPA. At day 10, colonies were selected (Fig. 1c). Colonies without VPA treatment tended to be much bigger than VPA-treated ones, their morphology not being typical of ESC colonies, although all colonies had cells positively expressing alkaline phosphatase staining (Fig. 1d). In contrast, VPA-treated colonies were more spherical and morphologically indistinguishable from ESC colonies (Supplementary Fig. 1). Cells of all ESC-like iPS colonies were positive for *Oct4* and *Nanog* expression (see below), indicating successful reprogramming of rat iPS-Cs from EF cells using the PB transposon system.

EF-derived FHH iPSCs had characteristics in common with rat ESCs

FHH rat iPSCs showed typical ES cell-like morphology with high nucleus-to-cytoplasm ratio, prominent nucleoli and clear boundaries of cells. They expressed SSEA-1, OCT4, NANOG and SOX2, as revealed by immunofluorescence staining (Fig. 2a). Data from RT-PCR analysis showed that endogenous pluripotency markers *Oct4*, *Sox2*, *Nanog*, and *Klf4* were successfully activated (Fig. 2b). Un-methylated state of the distal enhancer of *Oct4* expression reportedly is a feature of pluripotency in ESCs (17). To analyse DNA methylation states in rat iPSCs, we employed bisulphite-sequencing analysis of genomic regions within the distal enhancer of *Oct4* in the rat iPSCs, rat ESCs and EF cells. In the rat ESCs and iPSCs, both distal enhancers of *Oct4* were largely un-methylated. In contrast, in rat EF cells, the *Oct4* enhancer was highly methylated (Fig. 2c). Together, our

results indicate that rat iPSCs have ESC-like morphology, gene expression and epigenetic states.

FHH iPSCs differentiated into cells or tissues from all three germ layers in vitro and in vivo

To examine differentiation potential of FHH iPSCs *in vitro*, EB differentiation experiments were performed. The rat iPSCs formed well-shaped EBs (Fig. 3a). RT-PCR analysis of RNA samples from EBs on days 1, 4, 7 and 10 revealed gradual reduction in expression of endogenous pluripotency marker *Oct4* compared to expression in undifferentiated iPSCs. Simultaneously, expression of markers for major developmental lineages, including ectoderm (*Nestin*), mesoderm (*Kdr*) and endoderm (*Gata4*, *Sox17* and *Afp*), was activated (Fig. 3b). To test differentiation potential of rat iPSCs *in vivo*, they were injected into NOD/SCID mice to initiate teratoma formation. Four to five weeks later, teratomas were found to have formed. Histochemical analysis indicated existence of tissues from all three germ layers, such as pigment retinal epithelium (ectoderm), epidermal tissues (ectoderm), neural tube (ectoderm) (Fig. 3c), adipose tissue (mesoderm), muscle (mesoderm), undifferentiated mesenchymal cells (Fig. 3d), intestinal glands (endoderm) and cartilage (mesodermal) (Fig. 3e). Our results showed that the rat iPSCs could produce teratomas containing derivatives of three embryonic germ layers.

Contribution of rat iPSCs to somatic tissues and chimaeras

Before attempting to generate chimaeric rats, we evaluated karyotypes of FHH rat iPSCs; they were found to be normal (42 XY) (Table 2). To further test pluripotency of FHH rat iPSCs, we analysed their contribution to chimaera development. First, we had transfected FHH rat iPSC line with lentivirus-GFP and produced one GFP FHH rat iPS-2 cell line. About 185 F1 blastocysts

Figure 1. (a) Schedule of transposon-mediated reprogramming in rat EF cells in 2i or 3i culture system. (b) Morphology of rat EF cells before reprogramming using the transposon system. (c) Typical rat iPS clones after day 10. (d) Rat induced pluripotent stem cells (iPSCs) display AKP positivity.

Figure 2. (a) All colonies stained positively for Oct-4, Nanog, SSEA-1 and Sox2, and did not stain for SSEA-4. (b) RT-PCR detection of pluripotency-related genes and differentiation-related genes in mouse ES, rat ESCs, rat EF cells and rat embryos indicated that pluripotency-related genes all expressed in rat induced pluripotent stem cells (iPSCs), but not differentiation-related genes. (c) Bisulphite genomic sequencing of distal enhancer region of *Oct4*. Blank and Black circles indicate unmethylated and methylated CpGs, respectively.

Figure 3. (a) Embryoid bodies (EBs) were formed at Day 5. (b) Gene expression of three germ layers in embryoid bodies at different stages. Histopathological analyses of teratoma derived from rat induced pluripotent stem cells (iPSCs). (c) Primary neural tissue, pigment retinal epithelium (ectoderm), epidermal tissues and undifferentiated mesenchyme cells. (d) Lipocyte and muscle. E: Cartilage and glandular tissue.

(FHHN14 X SD) had been injected with FHH rat iPSCs (Fig. 4a) coming from 4 different rat iPSC lines. 5 chimaeras were produced (Fig. 4b). Previously, no germ-line transmission offspring were born. Forty blastocysts were injected with GFP-iPS-2 cells (Fig. 4c). Two GFP chimaeras were born with high FHH iPSC contribution (Fig. 4d) (Table 2). Microsatellite analyses (Fig. 4e) further confirmed presence of the FHH ESC genome in the resulting chimaeras. We concluded that the cultured cell genome was reproducibly contributing to the chimaeras. Our results showed that FHH rat iPSCs were competent to give rise to healthy adult chimaeras and to differentiate into fully functioning adult tissues of three germ layers. Although germ-line transmission in the adult chimaeras is

being analysed, we concluded that these FHH rat iPSCs were genuinely pluripotent. Hence, our results indicate that the PB transposon system reprogrammed pluripotent iPSCs from FHH rat EF cells.

Efficient targeting of p53 gene by routine homologous recombination in FHH rat iPSCs

To establish general applicability of gene targeting in rat iPSCs, we tried to knockout the *p53* gene via homologous recombination by electroporation. First, we finished the targeting vector that was designed to replace exon 2, 3, 4 and 5 with *PGK/Neo* cassette (Fig. 5a). The male FHH iPS-2 cell line, which was the source of genomic

Table 2. Chimaera rat generated from Fawn-Hooded Hypertensive (FHH) rat iPSCs

Cell line	iPS-1	iPS-2	iPS-3	iPS-4	GFP-iPS-2
Karyotype	42XY	42XY	42XX	42XY	42XY
Passage No.	P13	P16	P8	P5	P10
Host blastocyst	FHBN14XSD	FHBN14XSD	FHBN14XSD	FHBN14XSD	FHBN14XSD
Injected No.	56	45	37	48	40
Pups No.	13	19	6	24	8
Chimaeras No.	1(M)	2(1M,1F)	0	2(2F)	2(2F)
Germline No.	0	0	0	0	0

Figure 4. (a) Morphology of Fawn-Hooded Hypertensive (FHH) rat induced pluripotent stem cells (iPSCs), bright field. (b) Chimera contributed to by FHH rat iPSCs. (c) Morphology of GFP FHH rat iPSCs, bright field. (d) Fluorescence images of chimaeras with high contribution of GFP FHH iPSCs. (e) Microsatellite analyses of two adult coat colour chimaeras as contribution of FHH rat iPSCs. Polymorphic regions *D14Got23* and *D14rat7* amplified by PCR from genomic DNA of mouse ESCs, FHBN14, FHH ESCs, SD rat, wild type FHH rat, two chimaeras, and a littermate.

DNA for the homology arms in the targeting vector, was chosen for gene targeting experiments. The FHH rat iPSCs were transfected with linearized vector DNAs using a standard electroporation protocol, previously validated in rat ESCs (17). After approximately 10 days selection in medium containing aminoglycoside G418, the antibiotic-resistant ESCs were switched to medium containing FIAU for expanding. Resistant colonies indicated that the targeting vector was integrated correctly by homologous recombination. During selection, the FHH rat iPSCs retained their normal morphology, although some differentiated cells still existed (Fig. 5b). Southern blotting hybridized with 5', 3' and internal probes upstream of the homology arm confirmed accurate insertion of the targeting vector in FHH rat iPS-2 genome (Fig. 5c). Efficiency of *p53* targeting achieved with FHH rat iPSCs in our independent electroporation experiments is shown in Table 3. To confirm whether pluripotency was maintained in the targeted clones, we evaluated the stem-cell marker profile and *in vitro* differentiation capacity of *p53*-targeted clones. This analysis showed that RNA and protein expression patterns of the stem-cell markers

Oct4, Nanog, and more were similar to those of the parental cell line (data not shown). Our results indicated that FHH rat iPS-2 cells were amenable to gene targeting using routine homologous recombination and retained their pluripotent states. Furthermore, the *p53*-targeted iPSCs were karyotyped (Table 4) and normal karyotype iPSCs were selected for blastocyst injection. To date, we have injected 6 normal karyotype *p53*-targeted iPSC subcolonies; however, no of chimaera pups have yet been generated. Consequently, further analysis and more blastocyst injection will be necessary to prove whether or not these *p53*-targeted iPSCs were germ-line-competent.

Discussion

Here, we have showed that transduction with a transposon system carrying the five (*Oct4*, *Sox2*, *Klf4*, *Myc*, and *Lin28*: OSKML) or four (*Oct4*, *Sox2*, *Klf4*, and *Myc*: OSKM) reprogramming factor genes, and culture in the presence of two kinds of kinase inhibitors (MEK inhibitor and GSK-3 inhibitor) permitted efficient establishment and maintenance of rat iPSCs from FHH Rat

Figure 5. Schematic diagram illustrating the strategy to disrupt rat *p53* gene by homologous recombination in Fawn-Hooded Hypertensive (FHH) rat induced pluripotent stem cells (iPSCs). (a) Structures of wild-type (WT) rat *p53* allele and rat *p53* gene-targeting vector. S, *Sma* I; E, *Esp* I; black squares indicate exons. (b) Phase-contrast images of FHH rat iPSCs. Left images: before selection; right images: after selection. Scale bar, 50 μ m. (c) Southern blotting analysis of *p53* gene in targeted FHH rat iPSCs using 5', 3' and internal probes. Genomic DNA from FHH rat iPSCs was digested with *Sma* I or *Esp* I. For Southern blot analysis using neo probe, rat iPSC genomic DNA was digested with *Esp* I. Expected sizes of wild-type and *p53* gene targeted bands with different probes are shown.

EF cells. EF-derived FHH rat iPSCs possessed all the key features of rat ESCs, such as expression of pluripotency markers *Oct4* and *Nanog*, long-term self-renewal, capacity to differentiate into derivatives of all three germ layers and ability to produce high contribution chimeras. Most importantly, we also showed that the rat iPSCs supported homologous recombination using basic methodology as has been proven to be so effective in mouse and human ESCs (10, 18).

This study has a number of distinguishing characteristics. First, we established an efficient procedure to generate rat iPSCs from primary rat tissues. The main factor, to be perceived to affect successful generation of high contribution chimeras, is the condition under which rat iPSCs were generated and cultured. In recent studies (for example by Ping Li *et al.*), rat ESCs with germ-line competency were generated using serum-free N2B27 medium containing rat LIF and a combination of two or three kinase inhibitors (2i/3i) (11, 12). Rat iPSCs also have been generated using knockout DMEM medium containing knockout serum replacement (KSR), 2i, A83-01 and mouse LIF (19). As successful germ-line

Table 3. Frequency of routine homologous recombination in Fawn-Hooded Hypertensive (FHH) rat-induced pluripotent stem cells

Cell lines	Cell No.	G418 ^R	FIAU ^R	Frequency
FHH-iPS-2	7.43×10^6	101	35	4.71×10^{-6}
FHH-iPS-2	6.4×10^6	89	32	5.00×10^{-6}
FHH-iPS-2	5.51×10^6	76	30	5.45×10^{-6}
FHH-iPS-2	1.12×10^7	108	54	4.82×10^{-6}
FHH-iPS-2	4.4×10^6	67	27	6.14×10^{-6}
FHH-iPS-2	9.5×10^6	124	62	6.53×10^{-6}

transmission was produced in rat ESCs using N2B27 medium, we generated rat iPSCs using N2B27 medium and 2i/3i culture method, although Kawamata *et al.* also produced germ-line transmission using different media (7). Second, our results show that genetic manipulation can be achieved in rat iPSCs and importantly, the pluripotent state could be maintained after gene targeting. These strategies and methods described here will pave the way to obtaining disease model rats using iPS technology.

Table 4. The karyotype of *p53*-targeted induced pluripotent stem cells (iPSCs) subclones

Cell lines	Sub-clone	Passage	Karyotype ^a (%)	Metaphase counted
FHH-iPS-2	1	12	83.3	24
FHH-iPS-2	3	18	76.7	30
FHH-iPS-2	5	8	85.7	21
FHH-iPS-2	8	24	74.1	27
FHH-iPS-2	13	22	88.4	29
FHH-iPS-2	17	13	92	25
FHH-iPS-2	25	9	89.4	38

^aPercentage of metaphase containing euploid chromosome number of 42.

Assessment of reprogramming state of the somatic cell genome is essential for experiments in generation of iPSCs. The fully reprogrammed state is of practical importance as iPSCs are expected to differentiate accordingly into certain cell types. In our study, the rat iPSCs contributed highly to somatic cell content and formed teratomas with tissues originated from all three germ layers. Although germ-line transmission in adult chimaeras remains to be fully analysed, we concluded that these iPSCs are pluripotent.

The rat animal model is a critical experimental system for disease modelling, physiological, pharmacological and tissue behavioural studies in humans (11). Availability of competent rat ESCs and iPSCs will greatly facilitate and expand usefulness of the rat model for biomedical and translational research. Rat iPSCs are readily amenable to gene targeting by routine homologous recombination using basic methodology that has proven to be so effective in mouse and human ESCs. Efficiency of targeted gene insertion at the rat *p53* locus was similar to the first published reports for mouse and human ESCs (20–22). This demonstrates that rat iPSCs derived and maintained in 2i conditions are readily amenable to genetic modification, and this knowledge should encourage efforts to achieve routine gene targeting in rats. Important next steps in achieving this aim will be to refine culture methods to maximize iPSCs integrity, and to identify stem cell/embryo strain combinations that ensure reliable and efficient transmission of genetically modified cells through the germ line. Availability of *p53*-deficient rat iPSC lines also provides reagents with which to develop reliable, controllable transgene expression in rat iPSCs, as well as to accelerate the prospect of using large-scale genome engineering to humanize specific chromosomal regions in the rat.

In summary, FHH rat iPSC lines were generated from EF cells using a transposon system. The study demonstrated that the PB transposon-based method offered an alternative strategy for efficient generation of

rat iPSCs. At the same time, these rat iPSCs can be used for gene targeting. We envision that these strategies and methods will facilitate generation of disease-specific iPSCs and transgenic animals for drug discovery and cell-based regenerative medicine. Thus, we expect that rat iPSCs, together with their ES cell counterparts, will provide invaluable experimental models for assessing efficacy and safety of new cell-based therapies for varieties of disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 (a) Colonies without Valproic acid (VPA) treatment scattered and displayed some differentiation cells. (b) VPA-treated colonies were more spherical and morphologically indistinguishable from undifferentiated ES cell colonies.