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Viable Fertile Mice Generated from Fully Pluripotent iPS Cells Derived from Adult Somatic Cells

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Abstract Previous studies demonstrated that induced pluripotent stem (iPS) cells could produce viable mice through tetraploid complementation, which was thought to be the most stringent test for pluripotency. However, these highly pluripotent iPS cells were previously reported to be generated from fibroblasts of embryonic origin. Achieving fully pluripotent iPS cells from multiple cell types, especially easily accessible adult tissues, will lead to a much greater clinical impact. We successfully generated high-pluripotency iPS cells from adult tail tip fibroblasts (TTF) that resulted in viable, full-term, fertile TTF-iPS animals with no obvious teratoma formation or other developmental abnormalities. Comparison of iPS cells from embryonic origin (MEF), progenitor cells (neural stem cells) or differentiated somatic cells (TTF) reveals that fully pluripotent developmental potential can be reached by each cell type, although with different induction efficiencies. This work provides the means for studying the mechanisms and regulation of direct reprogramming, and has encouraging implications for future clinical applications and therapeutic interventions.

Keywords iPS cells · Tail tip fibroblast (TTF) · Fully pluripotent · Tetraploid complementation · Viable mice

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Introduction

Induced pluripotent stem (iPS) cells have been considered an important potential resource with great therapeutic potential for clinical applications [1]. Recent studies demonstrated that similar to embryonic stem (ES) cells, iPS cells derived from mouse embryonic fibroblast (MEF) cells can produce viable, fertile mice through tetroploid complementation (4N), an assay considered to be the gold standard for testing pluripotency [2, 3], indicating that high-quality iPS cells can differentiate into virtually all cell types in a living body. However, a demonstration that fully pluripotent iPS cells can be generated from different starting cell types is critical, especially for adult somatic cells such as fibroblasts that are relatively easily accessible [4]. Mouse adult tail tip fibroblasts (TTFs), one type of differentiated somatic cell, were shown to be difficult to fully reprogram and previous attempts to generate iPS-TTF gave highly variable results. At best, these studies resulted in one report of chimera production with germline

transmission [5], and there was no evidence indicating these cells were reprogrammed for full pluripotency, as only early stage (dead, E9.5) embryos and no full term mice were found after tetraploid complementation assays were performed using these iPS-TTF cells [6]. We herein report the generation of 23 iPS cell lines derived from mouse adult cells using our previously reported methods [2] with forced expression of the four "Yamanaka factors" and an Oct4-enhanced green fluorescent protein (GFP) reporter gene for direct reprogramming. We also produced iPS cells from neural stem cells (NSC) [7]; data from previously generated iPS cells (MEF-iPS) [2] from MEF were used for comparison.

Materials and Methods

Cell Culture ES cells and iPS cells were cultured as previous described [2]. Tail tip fibroblast (TTF) cells were generated from tail tips of 3 to 4-week-old mice, either females with a C57/B6×129 S2 F1 genetic background containing one Oct4-GFP cassette or males with a C57/B6×DBA/2 F1 genetic background without the GFP cassette, both cultured in 10% FBS (Gibco) DMEM medium. The neural stem cells (NSC) were derived from the whole brain of one-week-old mice (C57/B6×DBA/2 F1) as previously described [8], and cultured in N2B27 medium supplemented with 10 ng/ml EGF and 10 ng/ml bFGF (R & D systems).

Embryonic bodies (EBs) were formed from undifferented iPS cells cultured in uncoated dishes without LIF and feeder cells. The neural progenitors appeared 7–10 days after day-four EBs were replated on 0.1% gelatin coated dishes cultured with N2B27 medium [8], while beating cells could be seen 6–10 days after replating day-four EBs on 0.1% gelatin coated dishes and cultured in 10% FBS DMEM medium.

Note that all animal operations followed 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 and the iPS cells were derived as described [2], except for NSC-iPSC generation. Briefly, the four pMXs-based retroviral vectors (pMX-Oct4, Sox2, Klf4, c-Myc) were introduced into plat-E cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours later, virus supernatants were collected and supplemented with 4 ug/ml polybrene (Sigma). TTF cells were incubated with virus-containing supernatants for 48 h, while NSC cells were incubated for another 24 h. Cell cultures were then split and replated on feeder cells with 2.5×10^3 cells per 35 mm dish, and cultured with 20% Knockout Serum Replacement (KOSR) medium *(Invitrogen, cat. no. 10828-028)*

Immunofluorescence Analysis and Alkaline Phosphatase Staining iPS 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). The cells were incubated with primary antibodies to SSEA1 (Chemicon), Oct4 (Santa Cruz), or Nanog (Chemicon) overnight at 4°C, followed by secondary antibody at room temperature for 1 h. Alkaline phosphatase (AP) staining was performed with BCIP/NBT Alkaline Phosphatase Colour Development Kit (Beyotime) following the manufacturer's instructions.

Teratoma Formation and Histological Analysis iPS cells without feeder cells were trypsinized and suspended at 1×10^7/ml. One hundred microliters of cell suspension was injected into the subcutaneous flanks of SCID mice. Four to five weeks later, the mice were sacrificed and teratomas were collected, fixed and sliced, and the sections were stained with hematoxylin and eosin.

RT-PCR and Quantitative PCR Analysis Total RNA was isolated from freshly obtained cells using TRIzol reagent (Invitrogen). First strand cDNA was synthesized using M-MLV Reverse Transcriptase (Promega) and oligo-dT (Promega) according to the manufacturer's instructions. RT-PCR was performed using standard procedures and the products were electrophoresed on a 2% agarose gel. QPCR was performed using SYBR green (Applied Biosystems) on an ABI 7500 instrument.

Diploid Blastocyst (2N) Injection and Tetraploid Embryo (4N) Complementation The procedures followed those previously reported [2]. In particular, the diploid or tetraploid embryos were injected with 10–15 iPS cells before the embryos were transferred into the CD-1 recipient females for further development.

Bisulfite Genomic Sequencing Genomic DNA was bisulfite-treated with the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Nanog upstream regulatory regions were amplified with nested primers [2]. The first round of PCR was performed using 95°C for 5 min; then 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 min. The second round of PCR was performed with 95°C for 5 min; then 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s; and finally 72°C for 5 min. The PCR products were cloned into pMD18-T vectors (Takara). Ten randomly selected clones were sequenced and analyzed.

Determination of SSLP by PCR Sequences for specific primer pairs to measure simple sequence length polymorphisms were found on the Mouse Genome Informatics website (http://www.informatics.jax.org/). DNA was extracted from tail tips of the mouse, heart, liver, lung, kidney, skin, brain, spleen or cell pellet. Products were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

Southern Blot Analysis Genomic DNA was extracted by the classic phenol-chloroform method to detect the viral integration of the four transgenes. Twenty-five micrograms of genomic DNA were digested with Bgl II (Takara) for transgenic c-Myc detection and with BamH1 (NEB) for transgenic Oct4, Sox2 and Klf4 detection. Digested DNA was electrophoresed in a 0.8% agarose gel overnight and transferred onto a positively charged nylon membrane (Millipore). Prehybrydization and hybrydization were performed at 67°C using radioactively labeled probes prepared using the Prime-a-Gene Labeling System (Promega). Full-length cDNAs of each factor were used as template for probe synthesis. Primers used to synthesize the probes were:

Oct4-Forward: TGGACACCTGGCTTCAGA;
Oct4-Reverse: AGCAGTGACGGGAACAGA;
Sox2-Forward: TGGAGACGGAGCTGAAGCC;
Sox2- Reverse: CCCTGGAGTGGGAGGAAGAG;
c-Myc-Forward: ACTCCGTACAGCCCTATTTC;
c-Myc- Reverse: TTCAGCTCGTTCCTCCTCT;
Klf4- Forward: AGGGAGACCGAGGAGTTCA;
Klf4- Reverse: GGTAGTGCCTGGTCAGTTCAT.

Microarray Analysis

Miroarray analysis was performed as previously reported [9]. Total RNA was extracted using TRIzol reagent (Invitrogen) from three replicates of each cell line, including ESC-C1 (a B6D2F1 background ES cell line from which we recently succeeded in generating live 4N-Comp animals), 4N-Comp capable iPS lines of three origins: MEF-iPS (IP14D-1, IP14D-101), TTF-iPS (IP26DT-115, IP6DT-2), NSC-iPS (IP14NDN-5), and from original MEF, TTF and NSC cells. RNA mass and size distribution were determined using the Agilent Bioanalyzer with RNA 6000Nano LabChips, and 2.5 ug of total RNA were used as initial template for biotin-labeled cRNA amplification with the GeneChip One-Cycle kit (Affymetrix). Fifteen micrograms per replicate of fragmented cRNA were 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-plots were 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. The expression profiles of selected key pluripotency marker genes were also plotted for comparison. The microarray data for each sample are available in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo).

Results

Generation of iPS Cell Lines

After induction of MEF, TTF and NSC, positive iPS clones were initially observed in one of the NSC iPS lines at 6 days after ectopic expression of the Yamanaka factors (Fig. 1a–d). The infected NSCs formed many round, compact clonal colonies, whereas there were a few clones observed at a later time (14 days post viral delivery) with the MEFs and TTFs. Alkaline phosphatase (AP) assays were performed after culturing the infected cells for 14 days. AP^+ clones were identified from each of the three original cell types (Table S1); relative to number of cells infected, AP^+ colony number was highest in NSC cells, and significantly lower for TTF compared to MEF cells. AP is a marker for early reprogramming [10], thus, we also looked for GFP expression in MEF and TTF clones using a GFP reporter driven by the Oct4 promoter (Table S1). The number of Oct4-GFP positive clones from TTFs was about one-third of that from MEFs (93±61.60 and 28±13.07 GFP positive clones from MEFs or TTFs, respectively).

Twenty-three iPS lines were generated from 23 clones picked at 12 to 28 days post-viral infection (Table 1; Fig. S1). Among these iPS cells, 5 were derived from NSCs and 18 from adult TTFs.

Characterization of the iPS Cell Lines

In vitro assays were performed to characterize these iPSC lines. Pluripotency marker genes such as Nanog, SSEA-1 and Oct4 were expressed (Fig. 2a; Fig. S2); real-time RT-PCR showed the activation of the endogenous copies of these genes while the introduced exogenous genes were silenced in stable iPSC lines (Fig. 2b; Fig. S3). All cell lines tested have predominantly diploid karyotypes with 40 normal chromosomes, and bisulfite sequencing of the whole Nanog gene revealed demethylation both in the promoter and in the coding regions of the gene (Figs. S4 & S5). After removing feeder cells and leukaemia inhibitory

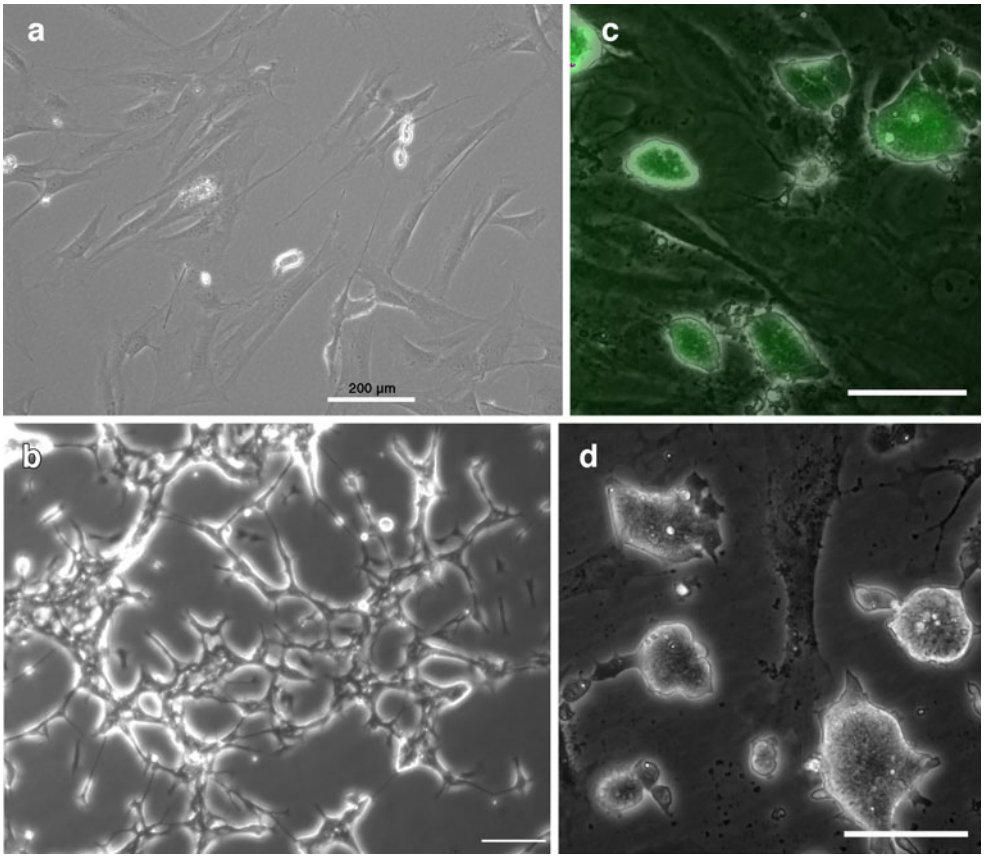

Fig. 1 Generation of iPS cells. **a**, Representative morphology of tail tip fibroblasts (TTF) from passage three; **b**, Representative morphology of neural stem cells on passage six; **c**, Fluorescence analysis of induced pluripotent stem cells carrying an Oct4-eGFP reporter gene (TTF-iPS); **d**, NSC induced pluripotent stem cells (NSC-iPS)

factor (LIF), these cells formed embroid bodies (EBs). Beating cells and neural progenitor cells appeared seven days after the EBs were placed in medium containing FBS, or in N2B27 medium[8], respectively (Fig. S6).

Generation of Chimeric Mice with Germline Transmission

To carry out in vivo pluripotency tests for these newly generated iPS cells, we first injected them into SCID mice. Three to four weeks later, teratomas were detected that were populated with cells from all three germ layers (Fig. 3). We also injected these iPS cells into normal CD-1 diploid blastocysts, and obtained high-quality chimeric mice (with 30–90% coat color chimerism for NSC-iPSCs and 30–70% for TTF-iPSCs, Table S2). After mating with CD-1 mice, both the NSC-iPS and TTF-iPS cells lines produced viable progeny through germline transmission, similar to the ES cells and MEF iPS cells previously reported [2].

Generation of Live-born iPS Mice Through Tetraploid Complementation

To further test the true pluripotency of the TTF-iPS cells, we performed the tetraploid complementation assay [2, 11]. The iPS cells were injected into tetraploid blastocysts and transferred into pseudo-pregnant CD-1 mice. Seventeen

Table 1 Summary of developmental efficiency for various iPS cell lines of different origins

Cell line	Genetic background	NO. of lines tested	Teratoma formation	Number of Cell Lines (No./Total Tested,%)			
				<10.5D	Embryos arrested at 10.5–13.5d	Embryos arrested at 15.5–17.5d	No. of live pups
TTF-iPS	C57x129S2	10	10	6 (60.0)	3 (30.0)	–	1 (10.0)
TTF-iPS	B6D2F1	8	8	1 (12.5)	0	–	7 (87.5)
NSC-iPS	B6D2F1	5	5	0	1 (20.0)	–	4 (80.0)
MEF-iPS	B6D2F1	4	4	2 (50.0)	–	–	2 (50.0)
MEF-iPS	C57x129S2	2	2	0	1 (50.0)	–	1 (50.0)
ESC	B6D2F1	5	5	2(40.0%)	–	–	3(60.0%)

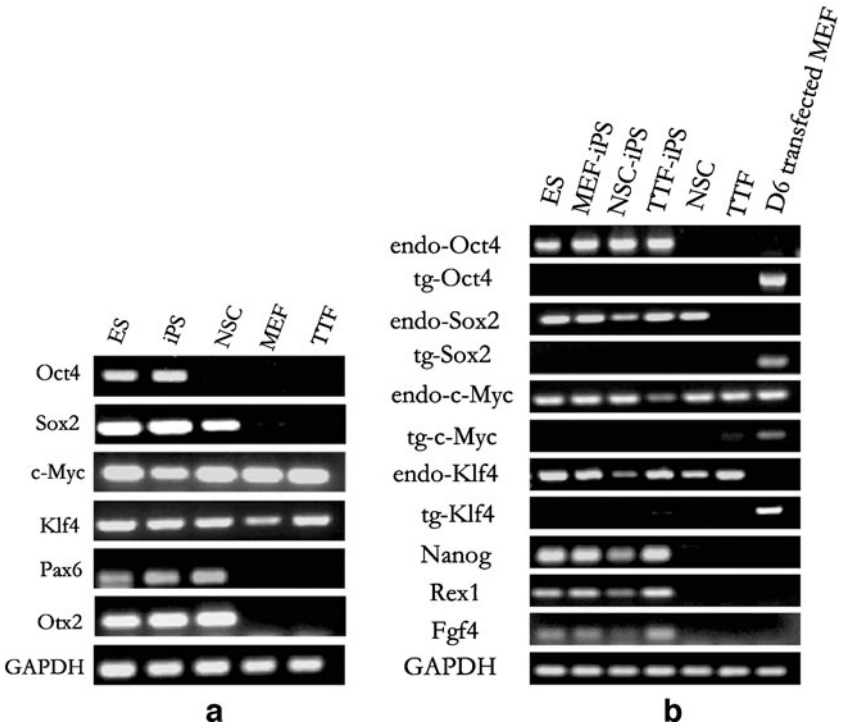

Fig. 2 RT-PCR analyses of several genes. **a**, RT-PCR analyses of RNA expression in ES, iPS (here one of the TTF-iPS was used as an example), NSC, MEF and TTF. GAPDH expression was used as a positive control **b**, RT-PCR analyses of pluripotency gene expression including endogenous Oct4, Sox2, c-Myc, Klf4 and the exogenous factors introduced during induction. GAPDH expression was used as a positive control

days later, iPS 4N-complemented (4N-comp) mice from both NSC-iPS and TTF-iPS cells were obtained. In particular, 4 out of 5 NSC-iPS cell lines (80%) generated live born NSC-iPS 4N-comp mice; the efficiency of generating live born pups ranged from 1.94% to 5.81% of total number of blastocysts injected. The remaining NSC-iPS cell line generated embryos that developed to E13.5 day with 2.93% efficiency (Table 1; Table S3). Among all 18 TTF-iPS cell lines, 10 have a C57×129S2 F1 genetic background originated from TTF of a female mouse and 8 have a BDF1 genetic background that were of male origin. Only one of the ten female lines was capable of producing live tetraploid mice, at a rate of 5% (of number of blastocysts injected), and this iPS line was derived at 26 days post retroviral infection. For the male TTF-iPS lines, seven of the eight TTF-iPS cell lines tested (87.5%) resulted in live births of TTF-iPS cell 4N-comp mice. These iPS lines were established by picking out clones at 16–17 days post infection and showed an efficiency of 0.82% to 6.86% for generating live, term 4N-comp mice. There

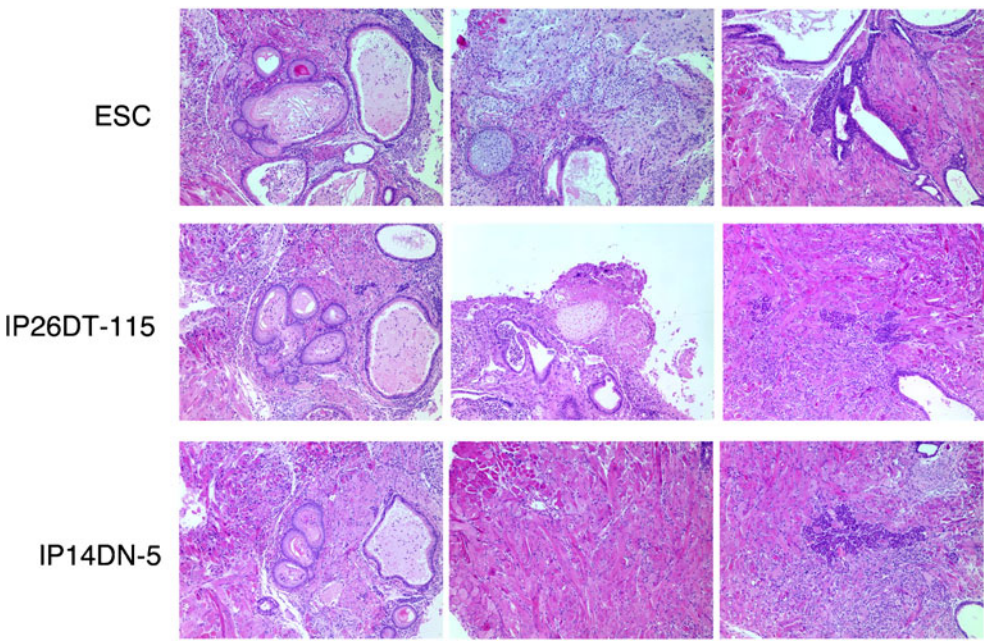

Fig. 3 Teratoma assay of iPS cells. Teratomas containing all three germ layers were observed for ES cells, TTF-iPS and NSC-iPS. Here results from IP26DT-115 and IP14DN-5 were used as examples for iPSC generated from TTF and NSC, respectively

were no significant differences among the ESC, NSC-iPS, MEF-iPS, and TTF-iPS in live birth efficiency (Table 1; Table S3), but the survival rate for the 4N-comp TTF-iPS mice seemed to be a little lower than for those generated from NSC, MEF or ESC (Fig. S7). The body weights of newborn 4N-comp mice from NSC-iPSCs and TTF-iPSCs were similar to those from the ESCs and MEF-iPS (Fig. S8). Note that all of the adult TTF-iPS 4N-comp mice and NSC-iPSC 4N-comp mice tested are fertile and can give birth to subsequent generations (Fig. 4a). Both the F0 (iPSC) mice and F1 (mated to CD-1) mice displayed no obvious teratoma formation from birth to adulthood.

Characterization of iPS Mice Derived Through Tetraploid Complementation

Simple sequence length polymorphism (SSLP) analyses and Southern blot analysis were used to confirm the lineage of the 4N-comp mice from the iPS cell lines that were generated (Fig. 4b; Figs. S9 & S10). The SSLP results consistently demonstrated that the 4N-comp mice came from their respective iPS cells without contribution from the host tetraploid blastocysts. Southern blot analysis also confirmed the origin of the 4N-comp mice, with similar integration patterns for the exogenous genes as observed in the initial iPS cells.

Global Gene Expression Analysis

Finally, global gene expression in iPS cell lines generated from different original sources was analyzed as previously reported [2, 9]. Hiearchical clustering analysis grouped all of the iPS cells with the ES cells in one distinct cluster, separated from the original MEF, TTF and NSCs (Fig. 5). As expected, pluripotency markers as well as other regulators were similarly expressed between the iPS and ES cells, but distinct from the initial cells from which they originated (Fig. S11). For example, the pluripotency marker genes Pou5f1 and Nanog were only found in the iPS and ES cells, but not the MEF, TTF, and NSCs; while Sox 2 was found in the iPS and ES cells as well as the NSC as expected, but not in MEF and TTF. Thus, consistent with the in vivo functional assays, the global expression patterns of 4N-comp iPS cells resemble ES cells, suggesting that irrespective of their origins and differences in efficiencies of induction, the resulting iPS cells achieve true pluripotency with a shared gene expression pattern.

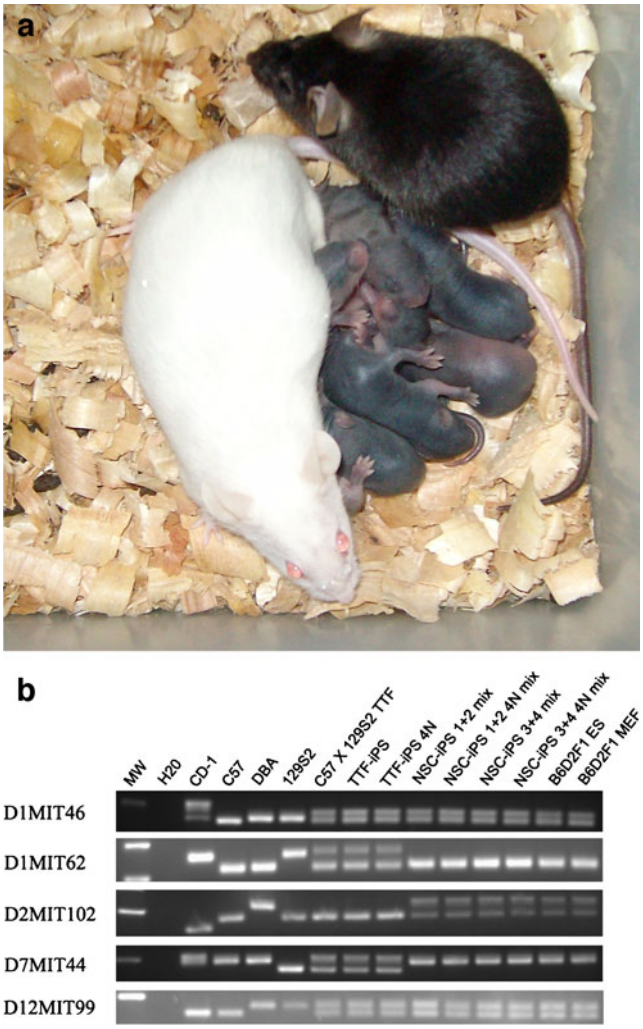

Fig. 4 Generation of iPS 4N-comp mice and lineage confirmation. **a**. Live 4N-comp mice were generated from TTF- and NSC-iPSC and raised to adulthood with normal maturation. Shown here is an example of an adult TTF-iPS (C57×DBA) 4N-comp mouse mated with a female CD-1 mouse, resulting in birth of viable next generation offspring. **b**. SSLP analysis for lineage confirmation of the origins of NSC-iPS and TTF-iPS. Note that the TTF-iPSC and NSC-iPSC mice have DNA polymorphism patterns similar to the parental cells from TTFs or NSCs, but distinct from the blastocyst host and foster mother CD-1 mice

Discussion

Establishing whether iPS cells hold the same developmental potential as ES cells is an important precondition for advancing pluripotent cell-based regenerative medicine and eventual clinical trials. Previous studies have already demonstrated that iPS cells could produce viable fertile mice through tetraploid complementation, which is considered to be the most stringent test for pluripotency [12, 13]. However, in these studies the truly pluripotent iPS cells originated from cells at embryonic stages. For clinical applications, the most compatible and convenient sources for iPS cells would be the patient's own adult tissues. Thus our confirmation that we can derive truly pluripotent iPS cells from adult cells is an important step towards development of clinical applications for iPS cells. Our

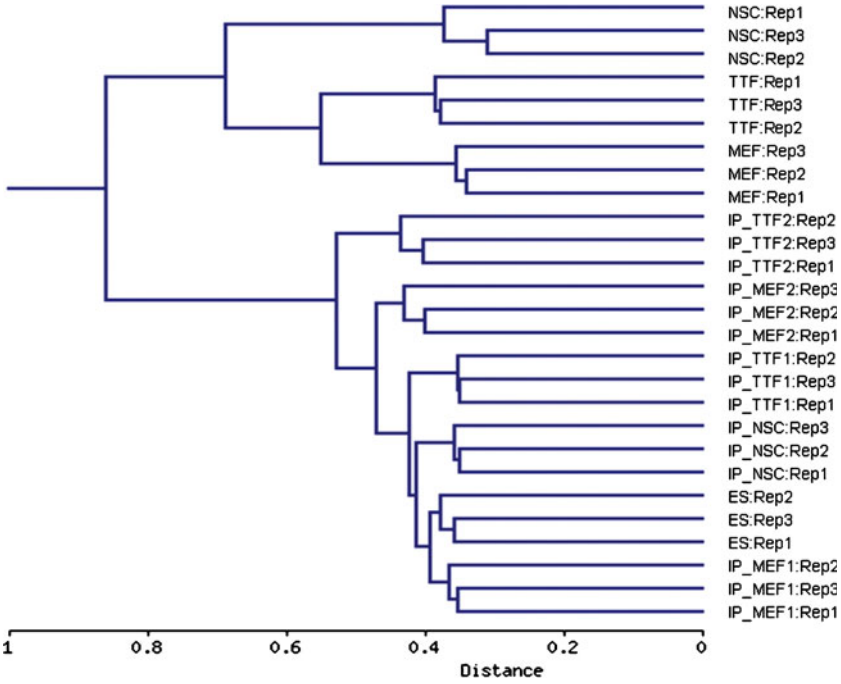

Fig. 5 Hierarchical clustering of gene expression patterns from 9 cell lines. Unsupervised hierarchical clustering was performed on whole-genome RNA expression patterns of 9 cell lines including: ES (ESC control, ESC-C1), IP_MEF1 (IP14D-1), IP_MEF2 (IP14D-101); IP_TTF1 (IP26DT-115), IP_TTF2 (IP16DT-2) and IP_NSC (IP14DN-5)

study also provides a useful model to explore mechanisms governing mammalian reprogramming. It has been well established that NSCs are more easily reprogrammed than MEFs as donors in nuclear transfer experiments, which is an oocyte-assisted reprogramming process [14]; in our experiments TTF appeared to be harder to reprogram, with a significantly lower efficiency of induction as well as requiring a longer time for reprogramming. These observations can be interpreted using Waddington's classical epigenetic landscape model which described cell fates being established during development much like a marble rolls down to the point of lowest local elevation [15]. Cell type differentiation can be seen as having increasing irreversibility as the cells travel down branching "valleys" of potential cell fates, separated by "ridges" formed by epigenetic and other molecular mechanisms. The reprogramming involved in iPS cells indicates that some differentiation pathways can, however, be reversed, and that reprogramming different cell types encounters different levels of resistance for going back "up the hill" (i.e. dedifferentiation). TTF may therefore have a "longer distance" and more resistance to dedifferentiation than do NSC. Here we showed that TTF derived iPS cells could generate viable mice, like ES cells or iPS cells from MEF or NSC cell origins. This clearly demonstrated that the iPS technology, though different from SCNT, can also be a powerful tool to reprogram adult cells from a differentiated state into a fully pluripotent state. Particularly, the in vivo developmental efficiencies for producing tetraploid complementation competent mice were comparable between ES cells and iPS cells from different cellular origins. This indicates that despite having different "distance" and "resistance" at the beginning of reprogramming, these cells may hold the same potential after they reach the fully pluripotent stage regardless of their cellular origins.

Our previous study using male MEFs showed little difference between genetic backgrounds for reprogramming efficiency, suggesting that the differences for TTFs observed here may be primarily influenced by donor gender rather than genetic strain [2]. Our observation of sex-linked differences is consistent with previous nuclear transfer experiments showing that female donor fibroblast cells are usually much harder to reprogram [16, 17], however, a further investigation comparing low and high efficiency lines from multiple cell sources within and between genetic backgrounds, and multiple lines from different genders of starting cells, may provide important insight into underlying epigenetic mechanisms reflected by the molecular signatures for pluripotency.

Conclusion

We have now successfully generated TTF-iPS cells that obtained complete pluripotency, as demonstrated by the tetraploid complementation assay. Viable, full-term, fertile TTF-iPS animals were generated with no obvious teratoma formation. Although the induction efficiency seemed to be reduced compared to iPS cells generated either from embryonic origins (MEF), or from progenitor cells (NSC),

the resulting TTF-iPS cells can be fully pluripotent and are capable of differentiation into all cell types, tissues and organs in a viable organism.

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Competing Financial Interests The authors declare no competing financial interests.

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