Tetraploid complementation proves pluripotency of induced pluripotent stem cells derived from adipose tissue

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Received 24 July 2014; revision accepted 21 August 2014

Abstract

Objectives: Recently, pluripotency of induced pluripotent stem (iPS) cells has been displayed after producing adult mice, in tetraploid complementation assays. These studies lead us to the last piece of the puzzle for reprogramming somatic cells into fully pluripotent cells which function as embryonic stem cells in most applications. However, in all of previous studies, skin fibroblasts were used as the starting population for reprogramming, raising questions as to whether the pluripotency of the iPS cells was dependent on the particular starting cell type.

Materials and methods: Our iPS cell lines were prepared from murine adipose stem cells (ASCs). Their multi-potency was first tested by teratoma formation in nude mice. Then, tetraploid complementation was performed to generate progeny from them.

Results: We succeeded to the birth of viable and fertile adult mice derived entirely from reprogrammed ASC, indicating cell types other than fibroblasts can also be restored to the embryonic level of pluripotency.

Conclusions: We also directed differentiation of iPS cells into chondrocytes, thus adipose-derived iPS cells can be used as models to study chondrogenic differentiation and cartilage regeneration.

Introduction

One of the most remarkable scientific findings of the 21st century was the discovery of four factors which

reprogram somatic cells into induced pluripotent stem (iPS) cells, by Takahashi *et al.* (1,2). By forced expression of the four defined transcription factors, cells from a patient could be induced into individual-specific or disease-specific iPS cells. This procedure involved selection for reprogrammed cells by activating endogenous pluripotency genes such as *Oct4* and *Nanog*, based on their morphological features (3,4). iPS cells derived from mouse or human fibroblasts are very similar to embryonic stem (ES) cells in their genetic, epigenetic and developmental characteristics. Thus, iPS cells hold great promise for future prospects of regenerative medicine and scientific research for fundamental aspect of human disease (5–7). However, most reported iPS cell lines have not been able to generate adult or full-term mice in tetraploid complementation assays (3,8,9), and gene expression differences have also been reported between iPS and ES cells (10). These data suggest that direct reprogramming may not be sufficient to restore mature cells to a fully totipotent level comparable to ES cells.

Some recent progress in this field has addressed this issue by generating full-term mice from iPS cell lines by tetraploid complementation, demonstrating that totipotency of iPS cells is, indeed, able to pass most stringent testing (11–14). Kang *et al.* used mouse embryo fibroblasts (MEFs) transduced with *rtTA* gene, and reported generation of one iPS cell line, capable of generating a complete iPS animal, through tetraploid complementation (14). Similar to this, Boland *et al.* used MEFs with an enhanced version of *rtTA* transcriptional activator protein combined with histone deacetylase inhibitor valproic acid, to generate tetraploid complementation competent iPS cells (13). Zhao *et al.* ectopically expressed four transcription factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) in MEFs to induce iPS, and selected positively reprogrammed cells by culturing transduced cells in medium containing knockout serum replacement (KOSR) without antibiotic selection (11). These findings are an important

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proof of principle. Nevertheless, all these studies were performed with fibroblasts. Thus, they cannot exclude the rare chance that production of full-term mice from iPS cell lines is a result of that particular cell type, as all used skin fibroblasts. It is still possible that these iPS cell lines have certain characteristics suitable for tetraploid complementation, but which may not be shared by lines derived from other cell sources.

To provide evidence to prove that pluripotency of iPS cells is not dependent on the cell type used for reprogramming, iPS cell lines must first be derived from adipose stem cells (ASCs) as these are reported to be an easily obtainable cell source that can be more efficiently reprogrammed into iPS cells (15,16). Then ASCs-derived iPS cells can be used to produce full-term mice through tetraploid complemented (Fig. 1).

Materials and methods

Cell culture

Adipose stem cells used for generation of ASiPS were isolated from inguinal fat pads of Oct4-GFP-labelled mice (B6D2F1 genetic background, that is, F1 of C57BL/6J3DBA/2J), by digestion of type I collagenase (0.075%; Sigma, St. Louis, MO, USA). ASCs were then cultured in medium containing α -MEM (Gibco, San

Diego, CA, USA), supplemented with 10% FBS (Gibco, San Diego, CA, USA) and penicillin and streptomycin (Gibco, San Diego, CA, USA). Immediately after viral transduction, infected ASCs were cultured in DMEM/F12 (1:1; Gibco, San Diego, CA, USA) and 20% knock-out serum (Gibco, San Diego, CA, USA). Established iPS cell lines and ES cell lines were cultured on mitomycin-C-treated MEF cells in PSC medium containing DMEM (Gibco, San Diego, CA, USA) plus 15% FBS, 1000 U/ml LIF (Chemicon, USA), 2 mM glutamine (Sigma, St. Louis, MO, USA), 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA), 0.1 mM β -mercaptoethanol (Sigma, St. Louis, MO, USA) and 0.1 mM non-essential amino acids. ES and iPS cells were then trypsinized and sub-cultured every 2 days. MEF cells for iPS/ES cell feeder layers were produced from E13.5 embryos with C573129S2 background.

All animals used for this study were guaranteed to be treated according to the 'Guidelines for Laboratory Animal Welfare' of Sichuan University.

Retroviral production and infection

Retroviral production and infection followed previously published protocols (17). Briefly, the four retroviral vectors (pMXs-Oct4, Sox2, c-Myc and Klf4) were introduced into plat-E cells using lipofectamine 2000 transfection reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Overnight after transfection, medium was replaced. Forty-eight hours later, virus-containing supernatants were collected and filtered through 0.45 mm filter (Millipore, Darmstadt, Germany), then supplemented with 4 mg/ml polybrene (Sigma, St. Louis, MO, USA). Oct4-GFP ASCs (seeded at 2×10^4 cells/cm²) were incubated with harvested supernatants for 24 h, following second infection for a further 24 h. Then virus-containing medium was replaced by regular ASCs media for 24 h. Three days after infection, transfected Oct4-GFP ASCs were sub-cultured on mitomycin-C-treated MEF feeder layers at 2×10^3 cells/cm², in induction medium (DMEM/F12, 1:1 and 20% knockout serum).

Immunofluorescence, immunohistochemical and alkaline phosphatase staining

Cells were fixed in 10% formalin for 30 min then permeabilized using 0.5% Triton X-100 for 30 min, followed by blocking with 1% BSA (Sigma). Cells were then incubated in primary antibody overnight at 4 °C, followed by secondary antibody incubation at room temperature for 1 h. Antibodies against Oct4 (Santa Cruz, USA), Sox2 (Chemicon, Darmstadt, Germany) and SSEA1 (Chem-

Figure 1. Schematic of production of tetraploid complementation mice from ASCs-derived iPS cells. The experimental procedure involved two phases: generation of iPS cells from ASCs, then their injection into tetraploid embryos to generate iPS mice. ASCs, adipose stem cells; iPS, induced pluripotent stem; hCG, human chorionic gonadotropin.

icon, Darmstadt, Germany) were used. For immunohistochemistry, paraffin wax sections were treated using routine procedures. Rabbit antibodies against COL I and COL II (both Abcam, Cambridge, MA, USA) were used, followed by incubation of HRP-conjugated secondary antibodies against rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Antibodies were then visualized using a peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA, USA). Alkaline phosphatase staining was performed with BCIP/NBT Alkaline Phosphatase Color Development kit (Beyotime, Nanjing, China) according to the manufacturer's protocols.

Teratoma formation and histology

Induced pluripotent stem cells were trypsinized and suspended at 1×10^7 per ml. One-hundred microlitres of the cell suspension was injected into subcutaneous flanks of severe combined immuno-deficient (SCID) mice. Four to five weeks later, the mice were euthanized and tumours were fixed and processed as for routine histological examination. 5 μ m sections were stained with haematoxylin and eosin.

Diploid blastocyst injection and tetraploid embryo complementation

Diploid blastocysts were gently flushed from uteri of E3.5 timed-pregnant mice, with CZB medium. Generation of mice by tetraploid embryo complementation was carried out as previously described in published protocols (17). Briefly, embryos at the two-cell stage were collected from oviducts of CD-1 females (coat colour, white), and electrofused to generate one-cell tetraploid embryos that were then cultured in CZB media. 10–15 iPS cells (originally from B6D2F1 genetic background - black coats) were injected into each tetraploid blastocyst and transferred to CD-1 pseudo-pregnant recipients. 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, Hilden, Germany) according to the manufacturer's protocols. Oct4 promoter regions were amplified with nested primers (Table S2). Both rounds of PCR were 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. Then, PCR products were cloned into pMD18-T vectors

(Takara, Dalian, China). Ten clones were randomly selected for sequencing and analysis.

Karyotype analysis

Karyotype analysis was conducted using standard murine chromosome analysis protocols.

Reverse transcription PCR

To test expression of pluripotent genes with endogenous and transgenic origin, total RNA was isolated using TRIzol reagent (Invitrogen). One microgram total RNA was reverse transcribed into cDNA in a 20 ml reverse transcription system (Fementas, Vilnius, Lithuania) according to the manufacturer's protocols. Then, cDNA samples were amplified using a Pfu PCR kit (Tiangen, Beijing, China). Primer sequences for each gene are listed in Table S1.

Embryoid body formation and generation of MSC-like cells

ASiPS were induced to form embryoid bodies (EBs) using the hanging drop method. Two days after hanging drop culture, EBs were transferred into petri dishes and maintained for 3 days in suspension culture in differentiation medium (iPS culture medium without LIF and in the presence of 10^{-7} M all-trans retinoic acid). After 3 days suspension culture, EBs were transferred to 0.1% gelatin-coated plates and cultured in the same medium for a further 3 days. Most EBs adhered and many cells migrated out from their edges. The following mesenchymal progenitors were sorted by FACS (fluorescence-activated cell sorting): CD326⁺, CD56⁺, CD73⁺, KDR⁺ and CD34⁺. Sorted cells were then sub-cultured in MSC growth medium, consisting of DMEM supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco).

Tri-lineage differentiation of mesenchymal progenitors

For adipogenesis, 50 000 mesenchymal progenitor cells were seeded in one well of a 12-well plate, with 1 ml of adipogenic medium, containing α -MEM plus 10% FBS, 10^{-6} M dexamethasone, 10 μ M insulin, 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 200 nM indomethacin, and 100 U/ml penicillin and 100 μ g/ml streptomycin. After 2 weeks induction, oil red O staining was performed to examine fat droplet formation. For osteogenesis, 40 000 mesenchymal progenitor cells were seeded in one well of a 12-well plate, with 1 ml of adipogenic medium, containing α -MEM plus 10% FBS, 10^{-8} M dexamethasone, 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate and 100 U/ml penicillin and 100 μ g/ml

streptomycin. Three weeks later, alizarine red staining was performed to reveal calcium deposition. For chondrogenesis, 50 000 cells per well were placed in a round bottomed 96-well plate with 200 µl chondrogenic medium, containing DMEM plus 40 µg/ml proline, 50 µg/ml ITS-premix, 50 µg/ml ascorbic acid, 100 µg/ml sodium pyruvate, 10 ng/ml TGF-β3 (transforming growth factor-β3), 10^{-7} M dexamethasone, and 100 U/ml penicillin and 100 µg/ml streptomycin. The plate was centrifuged at 500 g for 5 min to form aggregates. Three weeks later, aggregates were fixed and processed as for routine histology. Alcian blue staining was performed to detect glycosaminoglycans in the extracellular matrix.

Ectopic cartilage formation in nude mice

Pellets of 50 000 cells were made as described in the previous section. Chondrogenic differentiation was initiated by culturing pellets in chondrogenic medium for 1 week. Cell pellets were then implanted into subcutaneous flanks of SCID mice. Three weeks later, the mice were euthanized and implants were fixed and processed as for routine histological examination. 5 µm sections were stained with alcian blue, COL I and COL II antibodies (Abcam).

Results

Generation of induced-pluripotent stem cells from fat tissue

Stromal vascular fractions of white adipose tissue were separated from C57/BL6 mice by collagenase digestion,

to isolate proliferating mouse ASCs (ASCs). ASCs were further enriched by serial plate sub-culture and then infected with GFP-labelled retrovirus expressing four key factors (Oct4, Sox2, Klf4 and c-Myc). After 2 days, transduced cells were transferred on feeder cell layers from mouse embryonic fibroblasts (MEFs) and induced by culturing in medium with KOSR, but without antibiotic selection. After 10–15 days in KOSR induction medium, positive iPS clones were selected by morphology and GFP signal. Adipose stem cell-derived iPS cells (ASiPS) were then trypsinized and expanded over the next 6–8 days. Stable cell lines were cryopreserved and examined for karyotype and expression of pluripotent genes (Fig. 2a). ALP staining also confirmed that the ASiPS cell line was of pluripotent cells (Fig. S1).

Bisulphite sequencing was performed to examine methylation status of Oct4 promoters in the ASiPS lines. Compared to their parental ASCs, ASiPS had a different methylation pattern, closer to that of normal ES cells (Fig. 2b) reflecting epigenetic remodelling that occurred during reprogramming. RT-PCR analysis demonstrated that ASiPS lines expressed pluripotent marker genes *Oct4*, *Sox2*, *c-Myc* and *Klf4*, with a comparable expression pattern to that of ESC lines. Moreover, exogenous expression of these markers was observed to be silenced in ASiPS (Fig. 2c). This is similar to results of a previously performed study (3) in which transgenes were completely silent in the iPS cell lines, suggesting that maintenance of ASiPS lines mainly relies on endogenous expression of these four transcription factors. Immunofluorescent staining indicated expression of pluripotency markers Oct4, Sox2, SSEA1 and Nanog

Figure 2. Generation of adipose stem cells (ASCs)-derived induced pluripotent stem (iPS) cells and their characteristics. (a) Morphology of ASCs before and after viral transduction. Top panel, ASCs day 0 (uninfected), day 6, day 8 and after passaging; bottom panel, morphology of GFP⁺ cells day 6, day 8 and after passaging. Magnification for non-infected cells is 100×, original magnification, the rest being 400×. Bar = 50 µm. (b) Methylation analysis of Oct4 promoter regions. Genomic DNA from iPS cell lines at passage 10 as well as from ASCs and embryonic stem (ES) cells was isolated and bisulfite treated. Oct4 promoter regions were amplified with nested primers (Table S2). Ten clones were randomly picked for sequencing and analysing. Blank or filled circles represent unmethylated or methylated CpG dinucleotides, respectively. ASCs-derived iPS cell lines were quite different in methylation pattern from parental ASCs, but very close to those of normal ES cells, reflecting the epigenetic remodelling occurring together with reprogramming events. (c) RT-PCR confirmed that ASCs-derived iPS cells expressed both endogenous and transgenic ESC marker genes including *Oct4*, *Sox2*, *Myc* and *Klf4*.

Figure 3. Immunofluorescent staining of embryonic stem cells markers in adipose stem cells-derived induced pluripotent stem cells. Expression of Oct4 (purple), Sox2 (green) and SSEA1 (purple) was detected. DNA counterstained by propidium iodide (red). Scale bar = 50 μ m.

(Fig. 3) in ASiPS lines, while karyotypes assay confirmed the 40 chromosome content of our cell line to be normal (Fig. S2). Histological examination confirmed all three germ layers in the teratomas derived from the iPS cells injected into SCID mice (Fig. 4a,b), indicating a considerable level of pluripotency.

Tetraploid complementation of ASiPS

To test totipotency of the ASiPS, we injected these cells into tetraploid CD-1 blastocysts (white) for full-term development into pups. We observed complete development potential of ASiPS as indicated by the birth of live pups. Different iPS cell lines were not equally successful in producing viable offspring. A few cell lines formed foetuses whose development was terminated as early as embryonic days E 13.5 and E15.5. Figure 4c (left panel)

Figure 4. Teratoma formations and generation of induced pluripotent stem (iPS)-tetraploid mice. (a) Overview of severe combined immuno-deficient (SCID) mice and teratomas. (b) Sections stained with haematoxylin and eosin. Representative images from three germ layers are shown: ectoderm (epithelial tissue), mesoderm (muscle tissue) and endoderm (gland). Magnification 100 $\times$ original magnification. Bar = 50 μ m (c) Two pups from iPS cells (ASIP2-1-1) complemented with tetraploid blastocysts were born alive. (d) Two male pups generated from iPSCs tetraploid complementation grew to 2 weeks of age. These mice have uniformly black coats of S6D2F1 strain from which the adipose stem cells originated.

shows newly born pups generated from ASiPS. ASIP2-1-1 4N-comp pups have survived from 2 days to almost 9 months, at the time of writing. The two black mice in Fig. 4c right panel are representatives of the live iPS 4N-comp mice at 2 weeks. Uniform black coats of these mice is completely developed from iPS cells, similar to coat colour of the original line of ASCs (B6D2F1) from which they were derived.

ASiPS as models for cartilage regeneration

Induced pluripotent stem cells provide an excellent model for studying stem cell differentiation. We used a two-step differentiation protocol (Fig. 5a) to generate osteoblasts, chondrocytes and adipocytes from ASiPS

Figure 5. Differentiation of adipose stem cell-derived iPS (ASiPS) cells into muscle-skeletal lineages. (a) Schematic of generating osteoblasts, chondrocytes and adipocytes from multipotent ASiPS cells with two-step differentiation protocol. Dex, dexamethasone; β -GP, β -glycerophosphate; Sod Pyr, sodium pyruvate; IBMX, 3-Isobutyl-1- methylxanthine; Indo, Indomethacin. (b) MSCs migrated out of iPS embryo bodies. Bar = 200 μ m. (c) PSC-derived MSCs were induced to differentiate into adipocytes, osteoblasts and chondrocytes *in vitro*. Oil red O, alizarin red and alcian blue staining was performed to confirm the three lineage differentiation. Bar = 50 μ m. (d) Cell pellets were made from PSC-derived MSCs and cultured in chondrogenic medium for 1 week, then transplanted in nude mice subcutaneously for 3 weeks. Alcian blue staining was performed to indicate glycosaminoglycans, COL I and COL II expressions were also examined by immunohistochemistry. Bar = 50 μ m.

(18). ASiPS were first induced into mesenchymal progenitors. When embryonic bodies (EBs) were in adherent culture in MSC growth medium, cells with fibroblast-like morphology were observed to migrate out of them; these were ASiPS-MSCs (Fig. 5b). Then mesenchymal progenitors were sorted, based on expression of CD markers: CD326⁻, CD56⁺, CD73⁺, KDR⁻ and CD34⁻, by flow cytometry. After expansion *in vitro*, sorted mesenchymal progenitors differentiated into adipocytes, osteoblasts and chondrocytes under specific differentiation conditions (Fig. 5c). Implantation of mesenchymal progenitors in nude mice confirmed that the mesenchymal progenitors formed cartilage tissue *in vivo* (Fig. 5d).

Discussion

Viable live-born animals resulting from injection of diploid ES/iPS cells to create tetraploid (4N) embryos (blastocysts) were from diploid donor cells, as blastocysts from tetraploid hosts contribute only to extra-embryonic lineages, but not to the embryo itself (19,20).

Thus, tetraploid complementation showed ES/iPS cells used for transfection were able to develop into living animals; this is considered to be the most stringent test for pluripotency and developmental potency of embryonic/induced stem cells (21). Several reports recently have shown that full-term mice can be generated from iPS cell lines by tetraploid complementation, demonstrating totipotency of iPS cells (11–14). However, fibroblasts have been used as the starting population for making iPS cells in all these studies. Here however, we used adipose stem cell (ASCs)-derived iPS cells to produce full-term mice, through tetraploid complementation with the hope of testing the feasibility that production of full-term mice would be a result of certain characteristics suitable for tetraploid complementation from iPS cells derived from fibroblasts. Our data clearly demonstrate that the ASCs-derived iPS cell line was capable of producing 4N-comp mice.

Adipose stem cells are a type of mesenchymal stem cells that can be easily isolated in large quantities by lipoaspiration (22). ASCs are capable of differentiating into multiple lineages, including by osteogenesis, myo-

genesis, chondrogenesis and adipogenesis (23). Because of their plasticity, indicated by multilineage differentiation, it is believed that these cells have closer epigenetic regulatory pattern to pluripotent ES cells than terminally differentiated fibroblasts, which have been used in previous tetraploid complementation experiment. Such features in their epigenome have been proved to be an advantage for reprogramming, leading to higher efficiency and faster generation of iPS cells from ASCs (16). Other advantages of using ASCs as the starting population for reprogramming, include simple and fast isolation of ASCs with relatively lower donor morbidity, large quantities from a single procedure and independence of donor ages. As the most commonly used cells for generating iPS cells, skin fibroblasts are usually cultured *in vitro* for several weeks to obtained sufficient numbers for reprogramming after isolating them from a skin biopsy. However, amounts of ASCs derived from a single liposuction operation are more than enough for generating iPS cells. Thus, reprogramming experiments can be performed on the same day as liposuction, as viral transduction can be performed immediately after seeding stromal vascular fractions on culture plastic. Moreover, uninfected ASCs or ASCs not undergoing reprogramming can serve as feeder cells for the reprogrammed ones, as ASC feeder layers and ASC-conditioned medium have recently been reported to support expansion of hESCs (17,24). All these characteristics of ASCs are relatively independent on youth of the donors compared to other cell types used for reprogramming. Together with totipotency demonstrated here, ASCs-derived iPS cells are one of the most attractive cell sources for regenerative medicine.

In summary, we established ASiPS lines that displayed characteristics of ES cells in many aspects and one ASiPS line capable of producing 4N-comp mice. On the basis of data from the present and previous studies, we do not claim that iPS cells from all types of somatic cells can be reprogrammed to a pluripotent level equivalent to ES cells, however, we do provide evidence to prove that cells used for generation of totipotent iPS cells are not restricted to skin fibroblasts. Our data also indicate that ASiPS can be used to generate cartilage tissue *in vitro* and *in vivo*. ASiPS can be useful cell sources for regenerative medicine.

Acknowledgements

This work was funded by National Natural Science Foundation of China (31170929, 81201211), Sichuan Science and Technology Innovation Team (2014TD0001), and

Funding of State Key Laboratory of Oral Diseases (SKLOD201405).

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Supporting Information

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

Figure S1. ALP staining of ASiPS cell line.

Figure S2. Karyotype analysis of ASiPS cell line. One hundred individual chromosomal spread were analysis for each ASiPS cell sample. More than 75% of the cells showed normal mouse karyotype of 40 chromosomes, while representative image was shown.

Table S1. Primer sequence for real-time RT-PCR.

Table S2. PCR primer sequences for methylation studies.