Reprogramming Adipose Tissue-Derived Mesenchymal Stem Cells into Pluripotent Stem Cells by a Mutant Adeno-Associated Viral Vector

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Abstract

Induced pluripotent stem (iPS) cells have great potential for personalized regenerative medicine. Although several different methods for generating iPS cells have been reported, improvement of safety and efficiency is imperative. In this study, we tested the feasibility of using a triple tyrosine mutant AAV2 (Y444 + 500 + 730F) vector, designated AAV2.3m, to generate iPS cells. We developed a polycistronic rAAV2.3m vector expressing three reprogramming factors, Klf4, Oct4, and Sox2, and then used this vector to infect mouse adipose-derived mesenchymal stem cells (AT-MSCs) to induce the generation of iPS cells. We demonstrated that (1) the triple tyrosine mutant AAV2 vector is able to reprogram mouse adult adipose tissue-derived stem cells into the pluripotent state. Those rAAV2.3m-derived iPS (rAAV2.3m-iPS) cells express endogenous pluripotency-associated genes including Oct4, Sox2, and SSEA-1, and form teratomas containing multiple tissues *in vivo*; (2) *c-myc*, an oncogene, is dispensable in rAAV2.3m-mediated cellular reprogramming; and (3) transgene expression is undetectable after reprogramming, whereas vector DNA is detectable, indicating that transgenes are silenced. These results indicated the rAAV vector may have some advantages in generating iPS cells.

Introduction

STEM CELLS and induced pluripotent stem (iPS) cells hold great promise for regenerative medicine or cell-based therapy. Personalized or disease-specific iPS cells can be used for pathogenesis studies, drug screening, and gene corrections (Stadtfield and Hochedlinger, 2010). Mouse and human iPS cells have been established by transient overexpression of certain transcription factors (i.e., Oct4, Sox2, Klf4, and c-Myc), using various gene delivery vectors, including retrovirus, lentivirus, adenovirus, adeno-associated virus, Sendai virus, plasmid, and nonviral minicircle (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Okita *et al.*, 2008; Fusaki *et al.*, 2009; Sommer *et al.*, 2009; Zhou and Freed, 2009; Jia *et al.*, 2010; Weltner *et al.*, 2012b). Non-DNA-based methods have also been developed to increase the safety of iPS cells for clinical application, but these have shown lower reprogramming

efficiency (Zhou *et al.*, 2009; Cho *et al.*, 2010; Warren *et al.*, 2010). One of the potential problems with the DNA-based reprogramming method is the use of oncogenic transcription factor, c-Myc, to generate iPS cells. Studies have shown that tumor-associated death occurred in chimeras derived from iPS cells with c-Myc, and that mice derived from iPS cells that have not been transduced with c-Myc showed a significantly reduced incidence of tumorigenicity (Nakagawa *et al.*, 2008), suggesting the reactivation of the *c-myc* transgene may result in tumor formation (Okita *et al.*, 2007). These findings raise serious concern about the safety of iPS cells in clinical applications. Therefore, to make iPS cells safer for cell therapy, the elimination of c-Myc in cell reprogramming is highly recommended.

Adeno-associated virus (AAV) is a nonpathogenic parvovirus that has never been associated with any human diseases (Daya and Berns, 2008). Recombinant AAV (rAAV) vectors have been used extensively in gene therapy as a gene

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delivery tool and have been proven to be safe (Grieger and Samulski, 2012). Compared with other virus-based delivery methods, such as retrovirus, lentivirus, and adenovirus, rAAV carries no viral gene and has less immunogenicity toward transduced cells and human subjects (Daya and Berns, 2008). Studies on cell reprogramming kinetics demonstrate that transgene expression should be maintained for at least 12 days to obtain fully reprogrammed iPS cells (Brambrink *et al.*, 2008). Previous studies, including our research, showed that rAAV-mediated transgene expression could be sustained for several weeks in nondividing cells (Song *et al.*, 1998, 2001a). In addition, triple tyrosine mutant AAV2 vectors (Y444+500+730F) showed high infection efficiency toward primary murine bone marrow-derived mesenchymal stem cells (BM-MSCs) and fibroblasts (Li *et al.*, 2010). These features make the tyrosine mutant rAAV vector a good candidate to generate safer iPS cells.

Various cell types, including skin fibroblasts, keratinocytes, neural stem cells, CD34⁺ cord blood cells, bone marrow mononuclear cells (BM-MNCs), and adipose tissue-derived mesenchymal stem cells (AT-MSCs), have been tested and evaluated for cell reprogramming (Takahashi and Yamanaka, 2006; Kim *et al.*, 2009; Sun *et al.*, 2009; Aasen and Belmonte, 2010; Kunisato *et al.*, 2010; Takenaka *et al.*, 2010). Compared with other cell types, AT-MSCs have been shown to be compatible with embryonic stem (ES) cell culture medium and used as feeder layers for ES cell growth. Also, obtaining AT-MSCs is relatively less invasive and easy. Further, AT-MSCs, compared with IMR90 fibroblasts, showed an approximately 20-fold increase in reprogramming efficiency using four reprogramming factors (Sun *et al.*, 2009). These observations indicate that AT-MSCs may be a suitable starting cell type in developing novel cellular reprogramming methods. Taken together, the aim of the present study was to test the feasibility of using triple tyrosine mutant AAV2 vectors (Y444+500+730F), designated AAV2.3m, to generate iPS cells from mouse adipose tissue-derived mesenchymal stem cells in the absence of c-Myc.

Materials and Methods

Construction of pTR-AAV-CMV-mKOS vector

We designed an rAAV vector that can polycistronically express three mouse transcription factors (Klf4, Oct4, and Sox2), using the cytomegalovirus (CMV) promoter. The fragment, consisting of c-Myc-F2A-Klf4-T2A-Oct4-E2A-Sox2 (MKOS), was removed from plasmid pCAG2LMKOSimO (Addgene, Cambridge, MA) by restriction enzyme digestion, using *Bsa*BI. Then, this MKOS fragment was digested with *Dra*I and *Xba*I, and directly cloned into the *Hinc*II- and *Xba*I-digested pTR-CMV-AAT vector backbone. The resulting vector (pTR-CMV-c-Myc-F2A-Klf4-T2A-Oct4-E2A-Sox2) was further digested with *Xba*I and *Bst*BI to remove the c-Myc coding sequences, followed by a fill-in reaction with Klenow fragment to generate the plasmid pTR-AAV-CMV-Klf4-T2A-Oct4-E2A-Sox2 (pAAV-CMV-mKOS). In this vector, the reprogramming cassette is flanked by inverted terminal repeat (ITR) sites and therefore can be packaged into the rAAV vector. To generate iPS cells from AT-MSCs, we packaged the vector into a triple tyrosine mutant AAV2 capsid (Y444+500+730F) and designated it rAAV2.3m-CMV-mKOS.

Mouse AT-MSC isolation and feeder cell preparation

For a primary culture of AT-MSCs, a stromal vascular fraction (SVF) was freshly isolated from white adipose tissue of a human PiZ-hAAT transgenic mouse and a C57BL/6 mouse (6–8 weeks old). Adipose tissue was first enzymatically digested with 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) for 1 hr at 37°C with gentle agitation. The collagenase was then inactivated with an equal volume of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and the suspension was centrifuged at 1000×g for 5 min at room temperature. The resulting cell pellet was resuspended in 160 mM NH₄Cl, incubated at room temperature for 2 min to eliminate contaminating red blood cells, and filtered through a 100-μm nylon mesh strainer to remove debris. The resulting AT-MSC-containing cell pellet was collected by centrifugation as described previously, resuspended in DMEM–10% FBS medium, and plated on plastic tissue culture dishes. Adherent cells were cultured in DMEM–10% FBS medium supplemented with basic fibroblast growth factor (bFGF, 5 ng/ml) under hypoxic conditions (5% O₂ and 5% CO₂) for expansion (Yoshida *et al.*, 2009). For SNL76/7 feeder cells: SNL76/7 cells purchased from the American Type Culture Collection (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. To prepare the feeder layer, mitomycin C (10 μg/ml; Sigma-Aldrich) was added to the SNL76/7 cell culture medium, followed by incubation for 2 hr. These cells were then washed twice with PBS and replated onto a 6-well plate at a cell density of 2×10⁵ cells per well. It is recommended that mitomycin C-treated SNL76/7 feeder cells be used within 1 week.

Flow cytometric analysis

Fluorescence-activated cell-sorting (FACS) analysis of mouse AT-MSCs (passage 3) was carried out with either a BD LSRII analyzer (BD Biosciences, San Jose, CA) or an Accuri C6 flow cytometer (BD Biosciences) at the University of Florida (Gainesville, FL) FACS facility core. Antibodies used in this study were fluorescein isothiocyanate (FITC)-conjugated anti-CD90, -CD105, -CD31, and -CD45. All were purchased from eBiosciences (San Diego, CA). Dead cells stained with propidium iodide (PI) were excluded from the analysis.

Generation of iPS cells, using AT-MSCs

On the day before rAAV infection, AT-MSCs at an early passage (<passage 3) were seeded on a 6-well plate at a density of 6×10⁴ cells per well. The next day, AT-MSCs in serum-free medium were infected with rAAV2.3m-CMV-mKOS (multiplicity of infection [MOI], 10⁶). Six days after rAAV infection, the medium (DMEM containing 10% FBS) was replaced with ES cell specialized culture medium supplemented with 10% KnockOut serum replacement (KnockOut SR; Invitrogen, Carlsbad, CA), 1% FCS, 1% penicillin–streptomycin, 1% L-glutamine, 0.01% monothioglycerol (Sigma-Aldrich), leukemia inhibitory factor (LIF, 1000 U/ml), and vitamin C (50 μg/ml) (Esteban *et al.*, 2010). This was then incubated under hypoxic conditions as previously described (Yoshida *et al.*, 2009). Three to 5 weeks

after the induction, ES cell-like colonies were hand-picked and placed on feeder cells for expansion.

Immunocytochemistry and alkaline phosphatase staining

HEK293 cells grown on 24-well plates were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS, the cells were treated for 10 min with PBS containing 0.1% Triton X-100, followed by 1 hr of incubation with 1% bovine serum albumin (BSA) for blocking. The primary antibodies used were anti-Oct4 (diluted 1:400, C-10), anti-Sox2 (diluted 1:400, Y-17), anti-Klf4 (diluted 1:400, H-180), and anti-SSEA (stage-specific embryonic antigen)-1 (diluted 1:400, 480). All were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies used were rhodamine-conjugated goat anti-mouse IgG (diluted 1:400; Millipore, Billerica, MA), rhodamine-conjugated goat anti-mouse IgM (diluted 1:400; Millipore), FITC-conjugated donkey anti-rabbit IgG (diluted 1:400; Santa Cruz Biotechnology), and FITC-conjugated donkey anti-goat IgG (diluted 1:400; Santa Cruz Biotechnology). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Alkaline phosphatase staining was performed with an alkaline phosphatase staining kit (Stemgent, Cambridge, MA) in accordance with the manufacturer's instructions. In addition, primary antibodies used for teratoma immunostaining were as follows: anti- α -fetoprotein (AFP; Abcam, Cambridge, MA), α -smooth muscle actin (SMA; Abcam), and β_3 -tubulin (Santa Cruz Biotechnology). Staining with StainAlive™ SSEA-1 antibody (Stemgent) was performed according to the manufacturer's instructions.

Western blotting

Western blotting was performed as previously described (Takahashi *et al.*, 2007). Primary antibodies used were anti-Oct4 (diluted 1:500), anti-Sox2 (diluted 1:500), and anti-Klf4 (diluted 1:500). All were purchased from Santa Cruz Biotechnology. Secondary antibodies used in this study were horseradish peroxidase (HRP)-conjugated anti-mouse IgG (diluted 1:500) and HRP-conjugated anti-rabbit (diluted 1:500) (GE Health Life Sciences, Pittsburgh, PA), and HRP-conjugated anti-goat IgG (diluted 1:500; Santa Cruz Biotechnology).

Southern blotting

Total cellular DNA was purified from rAAV-iPS cells, using a DNeasy blood and tissue kit (Qiagen, Chatsworth, CA) and then subjected to Southern blotting analysis as described (Song *et al.*, 2001a). Briefly, 25 μ g of total cellular DNA was digested with or without *Xmn*I (a single cutter of the AAV vector). DNA fragments were separated by electrophoresis on a 0.8% agarose gel. Southern blot hybridization, using a 32 P-labeled DNA probe (CMV promoter, 599 bp), was performed at 60°C.

PCR and RT-PCR

Total DNA was purified with a Qiagen DNeasy blood and tissue kit according to the manufacturer's instructions. The vector-specific primers used in PCR to detect rAAV DNA were CMV-F (5'-CGTGTACGGTGGGAGGTCTATATAA-3') and CMV-R (5'-GGATCGGTCCCGGTGTCT-3'). For the

quantification of rAAV vector DNA in rAAV-iPSC clones, quantitative real-time PCR (qPCR) was performed with vector-specific primers and a probe for CMV promoter sequences: forward primer, 5'-CGTGTACGGTGGGAGGTCTATATAA-3'; reverse primer, 5'-GGATCGGTCCCGGTGTCT-3'; and probe, 5'-6FAM-ACGCCATCCACGCTGTTT GACCT-TAMRA-3' (Keeler *et al.*, 2012). In RT-PCR analysis, total RNA was purified with TRIzol reagent (Invitrogen). Total RNA (2 μ g) was used for the reverse transcription reaction using Qiagen Omniscript reverse transcriptase and oligo(dT) primers, according to the manufacturer's instructions. Real-time PCR was performed with SYBR green qPCR supermix (Invitrogen) and was analyzed with the DNA Engine Opticon 2 system (Bio-Rad, Hercules, CA). The primers used to detect endogenous reprogramming gene expression in real-time PCR were as follows: Endo Oct4-F (5'-CCAACGAGAAGAGTATGAGGC-3'), Endo Oct4-R (5'-GT GCTTTTAATCCCTCCTCAG-3'), Endo Sox2-F (5'-TCTGTG GTCAAGTCCGAGGC-3'), Endo-Sox2-R (5'-TTCTCCAGTT CGCAGTCCAG-3'), Endo-Klf4-F (5'-GGCGAGAAACCTT ACCACTGT-3'), Endo-Klf4-R (5'-TACTGAACTCTCTCTCC TGGCA-3'), Endo-c-Myc-F (5'-TCAAGCAGACGAGCACA AGC-3'), Endo-c-Myc-R (5'-TACAGTCCCAAAGCCCCAG C-3'), Endo-GAPDH-F (5'-AGGTCGGTGTGAACGGATT TG-3'), and Endo-GAPDH-R (5'-TGTAAGACCATGTAGTTG AGGTCA-3'). The primers used to detect vector-specific gene expression in RT-PCR were K2AO-F (5'-ACACCTGCGA ACTCACACAG-3') and K2AO-R (5'-AGTTGCTTTTCCACTC GTGCT-3'). The primers for GAPDH gene expression in RT-PCR are GAPDH-F (5'-ACCCAGAAGACTGTGGATGG-3'), and GAPDH-R (5'-CCCTGTTGCTGTAGCCGTAT-3').

Teratoma formation and examination

Approximately 9×10^6 rAAV2.3m-iPS cells were resuspended in DMEM containing 10% FBS and injected subcutaneously into C57BL/6 mice. Three weeks after injection, teratomas were dissected and fixed with 4% paraformaldehyde in PBS. Paraffin-embedded tissue was sliced and stained with hematoxylin and eosin (H&E). All the animal procedures including isolation of AT-MSCs and cell transplantation were approved by the University of Florida Institutional Animal Care and Use Committee (UF-IACUC).

In vitro differentiation

To generate embryoid bodies (EBs), rAAV2.3m-iPS cells were harvested by treatment with trypsin. The cells were transferred onto a Petri dish for growing bacteria and cultured in ES cell culture medium without LIF. After 5 days as a floating culture, EBs were collected and transferred to a gelatin-coated cell culture plate and cultured in the same medium for another 9 days.

Results

Isolation and characterization of adipose tissue-derived mesenchymal stem cells

Before reprogramming, we performed a detailed characterization of AT-MSCs. We isolated the stromal vascular fraction (SVF) from white adipose tissue of both mutant human α_1 -antitrypsin (PiZ-hAAT) transgenic mice and C57BL/6 mice. The initially adherent cells grew into spindle-

shaped cells that developed into visible colonies 1 week after plating on cell culture plates. The cells began to proliferate rapidly under hypoxic conditions (5% oxygen). After the third passage we examined lineage-specific cell surface markers by FACS (Fig. 1A). Among the suggested markers, proliferating mesenchymal stem cells exhibited CD90 (71.4%) and CD105 (70.7%), whereas they were negative for hematopoietic lineage markers, such as CD45 (<95%), or endothelial cell markers, such as CD31 (<95%). In comparison with mouse embryonic fibroblasts (MEFs), the most common starting cell type used in cell reprogramming, we showed that AT-MSCs have alkaline phosphatase activity (Fig. 1B) and that AT-MSCs expressed a low level of c-Myc and pluripotency-related genes, including Oct4, Sox2, and Klf4 (Fig. 1C). Consistent with previous findings (Zhu *et al.*, 2008), these observations suggested that AT-MSCs have a potential advantage as a starting cell type for generating iPSC cells.

Generation and evaluation of rAAV vectors expressing *Klf4*, *Oct4*, and *Sox2*

Previous studies suggested that generating iPSC cells requires reprogramming genes expressed for at least 12 days (Brambrink *et al.*, 2008). To test whether transgene expression kinetics from the rAAV vector met this requirement, we used a secretory protein (human α_1 -antitrypsin, hAAT) as a reporter and showed that rAAV vector-mediated transgene expression was sustained for up to 3 weeks (testing period) in nondividing cells and declined to a nearly undetectable

level in dividing cells after 8 days (Supplementary Fig. S1A and B; supplementary data are available online at <http://online.liebertpub.com/hgtb>). These kinetics profiles suggested that the rAAV vector has the potential to generate iPSC cells. To efficiently deliver reprogramming factors and avoid the potential problems of introducing multiple vectors into the same cell, we generated a single rAAV vector, pAAV-CMV-mKOS, which polycistronically expresses mouse *Klf4*, *Oct4*, and *Sox2* (Fig. 2A). In this vector, the CMV promoter drives the reprogramming cassette *Klf4-T2A-Oct4-E2A-Sox2*, which was originally derived from pCAG2LMKOSimO (Kaji *et al.*, 2009). Plasmid transfection of this vector showed that ES cell-specific proteins were highly expressed in HEK293 cells, indicating that the transgenes correctly expressed those specific proteins (Fig. 2B and C). To select an efficient rAAV serotype for the transduction of AT-MSCs, we infected AT-MSCs with several different rAAV vectors expressing green fluorescent protein (GFP), including rAAV1, 2, 5, and 7 and tyrosine mutant rAAV (rAAV2.3m and -2.6m). Results from these experiments showed that the tyrosine mutant AAV vectors mediated much higher transduction efficiency than did wild-type AAV vectors (Supplementary Fig. S1C). Consistent with observations from previous studies (Blin *et al.*, 2010; Li *et al.*, 2010), this finding indicated that tyrosine mutant AAV vectors have an advantage for gene delivery to AT-MSCs. Quantitative analysis by FACS also showed that rAAV2.3m infected AT-MSCs efficiently (Fig. 2D and E) and that transgene expression in AT-MSCs was sustained for up to 3 weeks (Supplementary Fig. S1D). On the basis of

FIG. 1. Characterization of adipose tissue-derived mesenchymal stem cells (AT-MSCs). **(A)** Representative FACS analysis histogram showing that mouse AT-MSCs expressed mesenchymal stem cell markers CD90 and CD105, but not hematopoietic lineage markers, such as CD45, or endothelial cell markers, such as CD31. **(B)** Alkaline phosphatase (AP) staining of AT-MSCs and mouse embryonic fibroblasts (MEFs) in cell culture dish. Some AT-MSCs expressed AP activity (*top*), whereas MEFs (*bottom*) did not. **(C)** RT-PCR analysis of endogenous gene expression of pluripotency-related genes and reprogramming factors in AT-MSCs and MEFs. Color images available online at www.liebertpub.com/hgtb

FIG. 2. Construction and evaluation of rAAV vectors. **(A)** Schematic map of the plasmid pAAV-CMV-mKOS; ITR, AAV inverted terminal repeat sequence; 2A, 2A peptide sequence of foot-and-mouth disease virus. **(B)** Immunostaining for mouse Oct4 and Sox2 in HEK293 cells transfected with pAAV-CMV-mKOS at 72 hr posttransfection. **(C)** Western blotting analysis for the detection of transgene products. Whole cell lysate of HEK293 cells transfected with pAAV-CMV-mKOS at 48 hr posttransfection was used. β -Actin was detected as a loading control. **(D)** Mouse AT-MSCs were infected with rAAV2.3m expressing GFP. **(E)** Percentages of GFP-positive cells were determined by flow cytometry. Color images available online at www.liebertpub.com/hgtb

previous results, we packaged our reprogramming vector into rAAV2.3m (rAAV2.3m-CMV-mKOS) to generate iPS cells from AT-MSCs.

Generation of induced pluripotent stem cells from adipose tissue-derived mesenchymal stem cells

Freshly isolated AT-MSCs (Fig. 3A) from C57BL/6 mice and PiZ-hAAT transgenic mice were infected with rAAV2.3m-CMV-mKOS. Six days after the infection the culture medium (DMEM containing 10% FBS) was replaced with ES cell culture medium containing leukemia inhibitory factor (LIF). Cell reprogramming was monitored by staining with StainAlive™ SSEA-1 antibody (Supplementary Fig. S2). Four weeks later, we observed distinct types of colonies that were flat and resembled mouse ES colonies (Fig. 3B). On day 30 we picked up mouse ES cell-like colonies and dissociated cell colonies by enzymatic digestion for further examination. We plated these ES-like cells on mitotically arrested SNL feeder cells with ES cell medium containing LIF and observed that they formed tightly packed and flat colonies (Fig. 3C, E, and G). The appearance of these cells was similar to that of mouse ES cells, characterized by large nuclei and scant cytoplasm. These cells showed feeder dependency at early passages, but can maintain an undifferentiated state on gelatin-coated tissue culture plates at later passages and are able to proliferate for at least 80 passages. Furthermore, they expressed ES cell-specific proteins, including Oct4 and Sox2, and specific surface antigens, such as stage-specific embryonic antigen

SSEA-1 (Fig. 3D, F, and H). In these experiments, feeder cells were used as a negative control to show the specificity of antibodies used (Supplementary Fig. S3). Because these cell clones were similar in appearance and markers compared with ES cells, we named them rAAV2.3m-derived iPS (rAAV2.3m-iPS) cells. To this end, the estimated iPS generation efficiency was 1–22 colonies for 6×10^4 cells as initial cell number (0.0016–0.036%).

Detection of rAAV DNA in rAAV2.3m-derived iPS cells

To test whether rAAV vector DNA persists in rAAV-derived iPS cells, total cellular DNA isolated from representative clones was subjected to Southern blotting analysis, using a 32 P-labeled CMV probe. When total cellular DNA was treated with no restriction enzyme (Fig. 4A, three left lanes), only a high molecular weight (HMW) band, but no low molecular weight (LMW) band, was detected, indicating that rAAV vector DNA was integrated into the cellular genome. When total cellular DNA was treated with *Xmn*I (a single cutter of the vector; Fig. 4B), the HMW band completely disappeared and two or three bands were detected (Fig. 4A, three right lanes). Because the sizes of the bands (8.5, 5.5, and 3.8 kb) were greater than that of the predicted vector fragment (3.5 kb) and did not match the predicted size of the head-to-tail (H-T, 4.7 kb) junction and head-to-head junction (H-H, 7.0 kb), these signals indicated that rAAV vector DNA integrated into two or three sites in the cellular genome.

To confirm rAAV DNA integration, we performed PCR-based analysis. Vector-specific PCR analysis showed that

FIG. 3. Characterization of rAAV2.3m-iPS cells. **(A)** Appearance of mouse AT-MSCs. **(B)** Representative image of mouse ES cell-like, rAAV2.3m-iPS cell colony. **(C–H)** Immunostaining for Oct4 (**C**, light view, **D**, dark view), Sox2 (**E**, light view, **F**, dark view), and SSEA-1 (**G**, light view, **H**, dark view). Color images available online at www.liebertpub.com/hgtb

rAAV DNA was detectable in rAAV2.3m-iPS cells (Fig. 5A). Quantitative genomic PCR (qPCR), using vector DNA-specific primers and probe, showed that vector DNA was detectable in all rAAV-iPSC clones at various passages (Supplementary Tables S1–S3). To eliminate the possibility of episomal DNA contamination, we passaged two clones to 14 and 18 passages. Quantitative PCR analysis again showed that rAAV vector DNA was detectable in all passages and clones (Supplementary Table S2). Because culture of rAAV-iPSCs at early passages requires feeder cells, those feeder cell DNAs were coisolated with the genomic DNA of rAAV-iPSCs, resulting in an underestimate of the copy numbers of rAAV vector DNA per rAAV-iPSC (Supplementary Tables S1 and S2). To avoid this limitation, we have passaged an iPSC clone to 20 passages, and isolated 5 subclones. These cells were cultured without feeder cells. As shown in Supplementary Table S3, all these subclones contained one or two copies of rAAV vector DNA per cell. Collectively, results from both Southern blot and PCR analysis showed that

rAAV viral DNA was integrated into the cellular genome in all our rAAV-iPSCs.

Characterization of rAAV2.3m-iPS cells

To test whether rAAV DNA was actively expressing the transgenes, we performed vector mRNA-specific RT-PCR analysis. Results from these experiments showed that transgene expression in rAAV-iPSCs was silenced (Fig. 5B). Real-time PCR analysis showed that rAAV2.3m-iPS cells maintained under feeder-free conditions expressed endogenous Oct4, Sox2, c-Myc, and Klf4 (Fig. 5C). To test the pluripotency of these rAAV2.3m-iPS cells *in vivo*, we transplanted rAAV2.3m-iPS cells (rAAV2.3m-iPS clones 01 and 02) into C57BL/6 mice. Three weeks after injection, we observed teratoma formation with comparable efficiency to mouse J1ES cells as control (Supplementary Fig. S4). Histological examinations showed that the teratoma contained various tissues (Fig. 6A–I), including neural epithelium (Fig.

FIG. 4. Southern blot analysis of molecular fate of the rAAV genome in rAAV2.3m-derived iPS cells. Total DNA (25 μ g) were purified from various iPS cell clones and incubated with no restriction enzyme (no cutter) or with *Xmn*I (one cutting site within the vector). **(A)** DNA was hybridized with a 32 P-labeled CMV probe. **(B)** Schematic diagram for restriction digestion and position of the probes (CMV) for hybridization. H-T junction, head-to-tail junction; H-H, head-to-head junction.

6C), gutlike epithelium (Fig. 6D), bonelike cells (Fig. 6E), and muscle-like cells (Fig. 6F). By immunostaining we detected cells expressing α -fetoprotein (AFP; endoderm marker), α -smooth muscle actin (SMA; mesoderm marker), and β_3 -tubulin (ectoderm marker) in the teratoma (Fig. 6G–I). In addition, we also evaluated the capability of these cells for *in vitro* differentiation. After 5 days of free-floating culture, rAAV2.3m-iPS cells began to aggregate and formed spherical clusters, so-called embryonic bodies (EBs). The EBs were then cultured on gelatin-coated plate in ES culture medium without LIF. After 2 weeks of culture, we performed immunostaining and detected cells positive for α -fetoprotein (Fig. 6J), α -smooth muscle actin (Fig. 6K), and β_3 -tubulin (Fig. 6L). These results indicate that rAAV2.3m-iPS cells are pluripotent and can be further differentiated into various tissues *in vitro* and *in vivo*.

Discussion

Recombinant adeno-associated virus (rAAV) has been widely used in preclinical and clinical studies and has been proven to be an effective and safe vector for the delivery of therapeutic genes to treat inherited disorders, such as Leber's congenital amaurosis (LCA) (Maguire *et al.*, 2008; Simonelli *et al.*, 2010), hemophilia B (Manno *et al.*, 2006; Nathwani *et al.*, 2011), and α_1 -antitrypsin (AAT) deficiency (Flotte *et al.*, 2011). There is no evidence showing that wild-type or recombinant AAV is associated with any human disease. One study reported that AAV2.3m shows relatively high infection efficiency toward murine bone marrow-derived mesenchymal stem cells and fibroblasts (Li *et al.*, 2010), which makes AAV2.3m a unique candidate vector for generating iPS cells. In the present study we successfully generated mouse iPS cells, using a single polycistronic AAV2.3m vector expressing three transcription factors: mouse Klf4, Oct4, and Sox2. The generated rAAV2.3m-iPS cells expressed endogenous ES

cell-specific proteins (such as Oct4, Sox2, and SSEA-1), formed teratomas in recipient mice, proliferated to at least 80 passages, and could be maintained under feeder-free (gelatin coating) culture conditions at later passages. More importantly, we showed that delivery of only three transcription factors is sufficient for AAV2.3m-mediated reprogramming and that transgene expression in rAAV2.3m-iPS cells is silenced. Considering that *c-myc* is an oncogene, generating iPS cells in the absence of c-Myc may be a great advantage in improving the safety of rAAV-derived iPS cells.

Studies have suggested that cell types that express high endogenous levels of particular transcription factors may have an advantage for cell reprogramming (Eminli *et al.*, 2008). We thus examined the gene expression profiles of isolated AT-MSCs by RT-PCR assay. Consistent with previous studies (Zhu *et al.*, 2008), we found that isolated AT-MSCs expressed endogenous Oct4, Sox2, Klf4, and c-Myc (Fig. 1C). This endogenous gene expression profile in isolated AT-MSCs may play an important role in promoting the generation of iPS cells by three transcription factors. In addition to endogenous expression of reprogramming genes, AT-MSCs have several additional advantages for generating iPS cells. For example, isolation, characterization, and handling of AT-MSCs *in vitro* are relatively simple; it has been shown that AT-MSCs can tolerate ES cell culture medium. Collectively, these observations suggest that AT-MSCs are excellent cell sources for generating iPS cells (Sun *et al.*, 2009; Aoki *et al.*, 2010). It should be noted that from our experience, in some experiments cell apoptosis was observed when older passages of AT-MSCs were used, probably due to incomplete epigenetic changes, which may lead to programmed cell death during the cellular reprogramming processes. This observation also highlights that the quality of isolated AT-MSCs is critical in rAAV2.3m-mediated cellular reprogramming.

Using three reprogramming factors to generate iPS cells has been achieved with retrovirus, but at lower efficiency

FIG. 5. (A) Detection of rAAV DNA in rAAV2.3m-iPS cells by DNA PCR. (B) Detection of vector-specific transgene expression in rAAV2.3m-iPS cells by RT-PCR. (C) Detection of endogenous gene expression profiles in rAAV2.3m-iPS cells by quantitative RT-PCR

(Nakagawa *et al.*, 2008). Generating iPS cells without c-Myc has not been reported in studies using other vectors, including adenovirus (Zhou and Freed, 2009), adeno-associated virus (Weltner *et al.*, 2012b), a plasmid-based method (Okita *et al.*, 2008), and nonviral minicircle DNA vectors (Jia *et al.*, 2010). In the present study we showed that delivery of three reprogramming factors in the absence of c-Myc by rAAV2.3m vector can generate iPS cells. One of the reasons for this success is the use of AT-MSCs as the starting cells. The low-level expression of Oct4, Sox2, Klf4, and c-Myc in mouse AT-MSCs (Fig. 1C) likely enhanced the cellular reprogramming processes, and thus exogenous genes, such as *c-myc*, could be eliminated.

Further improvement of reprogramming efficiency, such as by incubation with certain small molecules, may lower the required vector dose (or MOI) and thus increase the opportunity to derive rAAV-free iPS cells. Several studies have demonstrated that certain small molecules or drugs can enhance iPS induction efficiency (Li *et al.*, 2009; Chen *et al.*, 2010; Mali *et al.*, 2010; Yang *et al.*, 2011). These small molecules may participate in epigenetic processes, thereby overcoming epigenetic barriers during cellular reprogramming.

However, most of them are tested and evaluated in the context of fibroblast-based cellular reprogramming, and little is known about whether AT-MSCs tolerate these treatments or how well rAAV-transduced AT-MSCs react to small molecules during cellular reprogramming processes. It is therefore of great interest to investigate the interplay between these genetic factors and small molecules in rAAV2.3m-mediated cellular reprogramming. A better understanding of the mechanism underlying the rAAV-mediated cellular reprogramming process might help us identify participating factors in order to improve reprogramming efficiency.

In the present study, we found that AAV vector DNA was detectable within all generated rAAV2.3m-iPS cell clones (Figs. 4 and 5A, and Supplementary Table S1) regardless of passage number (Supplementary Table S2). At passages 5, 14, and 18, 0.12–1.48 copies of vector DNA were detected (Supplementary Tables S1 and S2). Because these cells were cultured with feeder cells and feeder cell DNA was co-harvested with rAAV-iPSC DNA, the rAAV vector DNA in Supplementary Tables S1 and S2 must have been underestimated. To eliminate feeder cell contamination, we cultured rAAV2.3m-iPS cells under feeder-free conditions to

FIG. 6. Pluripotency of mouse rAAV2.3m-iPS cells. (A) Isolated teratoma; (B) teratoma section (low magnification); (C) neural epithelium (ectoderm); (D) endodermal epithelium (endoderm); (E) bonelike cell (mesoderm); (F) muscle-like cells (mesoderm); (G and J) α -fetoprotein (endoderm marker); (H and K) α -smooth muscle actin (SMA) (mesoderm marker); (I and L) β_3 -tubulin (ectoderm marker); (B–F) H&E staining; (G–I) IHC staining for specific markers; (J–L) immunofluorescence staining. Color images available online at www.liebertpub.com/hgtb

passage 20. Analysis of DNA isolated from five subclones showed that the viral copy number per cell ranged from 1.25 to 1.95 (Supplementary Table S3). Consistent with previous observations described by Weltner and colleagues, who used four rAAV2 vectors to infect murine fibroblasts by multiple infections (Weltner *et al.*, 2012b), our results from Southern blotting and PCR-based analyses indicated that rAAV DNA integrated into the cellular genome of iPS cells. Together, these observations suggest a new paradigm concerning the molecular fate of rAAV genomes in host cells. In nondividing cells, rAAV DNA persists in host cells as episomal forms (linear or circular), which have been well documented and accepted (Duan *et al.*, 1998; Song *et al.*, 2001b; Schnepf *et al.*, 2003). However, in dividing cells, rAAV DNA may have two fates: (1) it can be diluted or lost as the host cell divides, which has been reported (Nakai *et al.*, 2001; Song *et al.*, 2004); or (2) rAAV DNA can also integrate into the cellular genome as the host cell divides. Although the proportions of rAAV DNA for each direction require complex detailed quantification, our data demonstrated that both events exist in dividing host cells. Recombinant AAV DNA integration has been reported under the selection conditions described (Nakai *et al.*, 1999). However, rAAV DNA integration in every iPS clone has not been observed until more recently

(Weltner *et al.*, 2012a). Weltner and colleagues used four rAAV2 vectors (each expressing one programming factor) and fibroblasts, whereas our study used one rAAV2.3 vector expressing three factors and AT-MSCs. The consistent observations from these two different studies suggest that rAAV integration in generating iPS cells is not vector or cell type dependent. It is possible that during the rAAV-mediated reprogramming process the rAAV-integrated cells gain prolonged transgene (reprogramming factors) expression and have the growth advantage over the cell clones that do not have the integrated transgene. Although different methods were used, results from our study and those of others raise important questions, including what critical factors attribute to the integration, the effects of the integration, and how to control or avoid the integrations. Although answering these questions requires further investigations, it is clear that generating iPS cells does not require vector DNA integration (Fusaki *et al.*, 2009; Zhou and Freed, 2009) and that rAAV DNA integration is controllable (McCarty *et al.*, 2004; Song *et al.*, 2004; Zhang *et al.*, 2007). In addition, carrying no viral or bacterial gene in rAAV vector can be considered an advantage for generating iPS cells.

Many studies have shown that rAAV can mediate long-term transgene expression *in vitro* and *in vivo* (Song *et al.*,

2001a; Daya and Berns, 2008). Surprisingly, we found that reprogramming gene expression was silenced in rAAV2.3m-iPS cells. It has been shown that the retroviral transgene is silenced in retrovirus-mediated cell reprogramming (Okita *et al.*, 2007), and that the timing of retroviral silencing correlates with the quality of induced pluripotent stem cell lines (Okada and Yoneda, 2011). Such retroviral transgene silencing may result from the activation of potent repressor factors or the reduction of certain activating factors after directly reprogramming somatic cells to pluripotent states (Hotta and Ellis, 2008). In addition, CMV promoter activity has been shown to be progressively silenced in pluripotent stem cells (Xia *et al.*, 2007; Meilinger *et al.*, 2009). In rAAV2.3m-mediated cell reprogramming, whether transgene silencing resulted from the activation of certain endogenous potent repressor or from the reduction of CMV promoter activity in rAAV-iPS cells remains elusive. Also, how the timing of AAV silencing will affect the quality of AAV2.3m-derived iPS cells need further investigation. Nonetheless, we believe that transgene silencing in rAAV2.3m-iPS cells increases the safety of using these generated iPS cells for cell transplantation purposes. Furthermore, our study provides a novel iPS cell experimental platform with which to investigate the epigenetic mechanisms of rAAV vector silencing, and to identify specific endogenous proteins in pluripotent stem cells that may specifically suppress rAAV-mediated transgene expression after reprogramming.

In summary, we have shown in this study that triple tyrosine mutant AAV2 vectors can reprogram mouse adipose tissue-derived mesenchymal stem cells into the pluripotent state in the absence of c-Myc. The transgene expression was silenced after reprogramming, although rAAV DNA was detectable in iPS cells. Considering the well-known safety features of rAAV vectors, this rAAV2.3m-mediated cell reprogramming method may provide a novel path for enhancing the application of iPS cells. Further studies are recommended to derive rAAV-free iPS cell clones.

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Author Disclosure Statement

No competing financial interests exist.

References

- Aasen, T., and Belmonte, J.C. (2010). Isolation and cultivation of human keratinocytes from skin or plucked hair for the generation of induced pluripotent stem cells. *Nat. Protoc.* 5, 371–382.
- Aoki, T., Ohnishi, H., Oda, Y., *et al.* (2010). Generation of induced pluripotent stem cells from human adipose-derived stem cells without c-MYC. *Tissue Eng. Part A* 16, 2197–2206.
- Blin, G., Nury, D., Stefanovic, S., *et al.* (2010). A purified population of multipotent cardiovascular progenitors derived from primate pluripotent stem cells engrafts in postmyocardial infarcted nonhuman primates. *J. Clin. Invest.* 120, 1125–1139.
- Brambrink, T., Foreman, R., Welstead, G.G., *et al.* (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. *Cell Stem Cell* 2, 151–159.
- Chen, T., Yuan, D., Wei, B., *et al.* (2010). E-cadherin-mediated cell–cell contact is critical for induced pluripotent stem cell generation. *Stem Cells* 28, 1315–1325.
- Cho, H.J., Lee, C.S., Kwon, Y.W., *et al.* (2010). Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. *Blood* 116, 386–395.
- Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. *Clin. Microbiol. Rev.* 21, 583–593.
- Duan, D., Sharma, P., Yang, J., *et al.* (1998). Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.* 72, 8568–8577.
- Eminli, S., Utikal, J., Arnold, K., *et al.* (2008). Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression. *Stem Cells* 26, 2467–2474.
- Esteban, M.A., Wang, T., Qin, B., *et al.* (2010). Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell* 6, 71–79.
- Flotte, T.R., Trapnell, B.C., Humphries, M., *et al.* (2011). Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing α_1 -antitrypsin: Interim results. *Hum. Gene Ther.* 22, 1239–1247.
- Fusaki, N., Ban, H., Nishiyama, A., *et al.* (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 85, 348–362.
- Grieger, J.C., and Samulski, R.J. (2012). Adeno-associated virus vectorology, manufacturing, and clinical applications. *Methods Enzymol.* 507, 229–254.
- Hotta, A., and Ellis, J. (2008). Retroviral vector silencing during iPS cell induction: An epigenetic beacon that signals distinct pluripotent states. *J. Cell Biochem.* 105, 940–948.
- Jia, F., Wilson, K.D., Sun, N., *et al.* (2010). A nonviral minicircle vector for deriving human iPS cells. *Nat. Methods* 7, 197–199.
- Kaji, K., Norrby, K., Paca, A., *et al.* (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 458, 771–775.
- Keeler, A.M., Conlon, T., Walter, G., *et al.* (2012). Long-term correction of very long-chain acyl-CoA dehydrogenase deficiency in mice using AAV9 gene therapy. *Mol. Ther.* 20, 1131–1138.
- Kim, J.B., Zaehres, H., Arauzo-Bravo, M.J., and Scholer, H.R. (2009). Generation of induced pluripotent stem cells from neural stem cells. *Nat. Protoc.* 4, 1464–1470.
- Kunisato, A., Wakatsuki, M., Kodama, Y., *et al.* (2010). Generation of induced pluripotent stem cells by efficient reprogramming of adult bone marrow cells. *Stem Cells Dev.* 19, 229–238.
- Li, M., Jayandharan, G.R., Li, B., *et al.* (2010). High-efficiency transduction of fibroblasts and mesenchymal stem cells by tyrosine-mutant AAV2 vectors for their potential use in cellular therapy. *Hum. Gene Ther.* 21, 1527–1543.
- Li, W., Zhou, H., Abujarour, R., *et al.* (2009). Generation of human-induced pluripotent stem cells in the absence of exogenous Sox2. *Stem Cells* 27, 2992–3000.

- Maguire, A.M., Simonelli, F., Pierce, E.A., *et al.* (2008). Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248.
- Mali, P., Chou, B.K., Yen, J., *et al.* (2010). Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. *Stem Cells* 28, 713–720.
- Manno, C.S., Pierce, G.F., Arruda, V.R., *et al.* (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat. Med.* 12, 342–347.
- McCarty, D.M., Young, S.M., Jr., and Samulski, R.J. (2004). Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu. Rev. Genet.* 38, 819–845.
- Meilinger, D., Fellinger, K., Bultmann, S., *et al.* (2009). Np95 interacts with *de novo* DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. *EMBO Rep.* 10, 1259–1264.
- Nakagawa, M., Koyanagi, M., Tanabe, K., *et al.* (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotechnol.* 26, 101–106.
- Nakai, H., Iwaki, Y., Kay, M.A., and Couto, L.B. (1999). Isolation of recombinant adeno-associated virus vector–cellular DNA junctions from mouse liver. *J. Virol.* 73, 5438–5447.
- Nakai, H., Yant, S.R., Storm, T.A., *et al.* (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *J. Virol.* 75, 6969–6976.
- Nathwani, A.C., Tuddenham, E.G., Rangarajan, S., *et al.* (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N. Engl. J. Med.* 365, 2357–2365.
- Okada, M., and Yoneda, Y. (2011). The timing of retroviral silencing correlates with the quality of induced pluripotent stem cell lines. *Biochim. Biophys. Acta* 1810, 226–235.
- Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448, 313–317.
- Okita, K., Nakagawa, M., Hyenjong, H., *et al.* (2008). Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 322, 949–953.
- Schnepf, B.C., Clark, K.R., Klemanski, D.L., *et al.* (2003). Genetic fate of recombinant adeno-associated virus vector genomes in muscle. *J. Virol.* 77, 3495–3504.
- Simonelli, F., Maguire, A.M., Testa, F., *et al.* (2010). Gene therapy for Leber's congenital amaurosis is safe and effective through 1.5 years after vector administration. *Mol. Ther.* 18, 643–650.
- Sommer, C.A., Stadtfeld, M., Murphy, G.J., *et al.* (2009). Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells* 27, 543–549.
- Song, S., Morgan, M., Ellis, T., *et al.* (1998). Sustained secretion of human α -1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14384–14388.
- Song, S., Embury, J., Laipis, P.J., *et al.* (2001a). Stable therapeutic serum levels of human α -1 antitrypsin (AAT) after portal vein injection of recombinant adeno-associated virus (rAAV) vectors. *Gene Ther.* 8, 1299–1306.
- Song, S., Laipis, P.J., Berns, K.I., and Flotte, T.R. (2001b). Effect of DNA-dependent protein kinase on the molecular fate of the rAAV2 genome in skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4084–4088.
- Song, S., Lu, Y., Choi, Y.K., *et al.* (2004). DNA-dependent PK inhibits adeno-associated virus DNA integration. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2112–2116.
- Stadtfeld, M., and Hochedlinger, K. (2010). Induced pluripotency: History, mechanisms, and applications. *Genes Dev.* 24, 2239–2263.
- Sun, N., Panetta, N.J., Gupta, D.M., *et al.* (2009). Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15720–15725.
- Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676.
- Takahashi, K., Tanabe, K., Ohnuki, M., *et al.* (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872.
- Takenaka, C., Nishishita, N., Takada, N., *et al.* (2010). Effective generation of iPS cells from CD34⁺ cord blood cells by inhibition of p53. *Exp. Hematol.* 38, 154–162.
- Warren, L., Manos, P.D., Ahfeldt, T., *et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7, 618–630.
- Weltner, J., Anisimov, A., Alitalo, K., *et al.* (2012a). Induced pluripotent stem cell clones reprogrammed via recombinant adeno-associated virus-mediated transduction contain integrated vector sequences. *J. Virol.* 86, 4463–4467.
- Weltner, J., Anisimov, A., Alitalo, K., *et al.* (2012b). iPSC clones reprogrammed via rAAV-mediated transduction contain integrated vector sequences. *J. Virol.* 86, 4463–4467.
- Xia, X., Zhang, Y., Zieth, C.R., and Zhang, S.C. (2007). Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner. *Stem Cells Dev.* 16, 167–176.
- Yang, C.S., Lopez, C.G., and Rana, T.M. (2011). Discovery of NSAID and anticancer drugs enhancing reprogramming and IPS cell generation. *Stem Cells* 29, 1528–1536.
- Yoshida, Y., Takahashi, K., Okita, K., *et al.* (2009). Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 5, 237–241.
- Zhang, C., Cortez, N.G., and Berns, K.I. (2007). Characterization of a bipartite recombinant adeno-associated viral vector for site-specific integration. *Hum. Gene Ther.* 18, 787–797.
- Zhou, H., Wu, S., Joo, J.Y., *et al.* (2009). Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 4, 381–384.
- Zhou, W., and Freed, C.R. (2009). Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem Cells* 27, 2667–2674.
- Zhu, Y., Liu, T., Song, K., *et al.* (2008). Adipose-derived stem cell: A better stem cell than BMSC. *Cell Biochem. Funct.* 26, 664–675.

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