Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors

Kazim H Narsinh[1,2,6], Fangjun Jia[1,6], Robert C Robbins[3], Mark A Kay[4], Michael T Longaker[3,5] & Joseph C Wu[1,5]

[1]Departments of Medicine and Radiology, Stanford University School of Medicine, Stanford, California, USA. [2]University of California San Diego School of Medicine, La Jolla, California, USA. [3]Department of Surgery, Stanford University School of Medicine, Stanford, California, USA. [4]Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, California, USA. [5]Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California, USA. [6]These authors contributed equally to this work. Correspondence should be addressed to J.C.W. (joewu@stanford.edu).

Published online 23 December 2010; doi:10.1038/nprot.2010.173

Human induced pluripotent stem cells (hiPSCs) derived from patient samples have tremendous potential for innovative approaches to disease pathology investigation and regenerative medicine therapies. However, most hiPSC derivation techniques use integrating viruses, which may leave residual transgene sequences as part of the host genome, thereby unpredictably altering cell phenotype in downstream applications. In this study, we describe a protocol for hiPSC derivation by transfection of a simple, nonviral minicircle DNA construct into human adipose stromal cells (hASCs). Minicircle DNA vectors are free of bacterial DNA and thus capable of high expression in mammalian cells. Their repeated transfection into hASCs, abundant somatic cell sources that are amenable to efficient reprogramming, results in transgene-free hiPSCs. This protocol requires only readily available molecular biology reagents and expertise, and produces hiPSC colonies from an adipose tissue sample in ~4 weeks.

INTRODUCTION

Human induced pluripotent stem cells (hiPSCs) can be derived from somatic cells through a reprogramming process driven by overexpression of a defined set of transcription factors[1,2]. These hiPSCs share the properties of self-renewal and pluripotency with human embryonic stem cells (hESCs), and can therefore be used to generate unlimited quantities of differentiated cell types of all three germ layers, including cardiac cells, neural cells and hepatic cells. hiPSCs can be generated from patients of virtually any genetic background, including those with disease-conferring genetic mutations[3–5]. In contrast, the derivation of hESCs from different genetic backgrounds is challenging because human embryo use is limited and ethically debated. The production of patient-specific and disease-specific hiPSCs enables a variety of downstream applications, including drug screening, disease modeling, pathogenesis studies and regenerative medicine therapies.

However, traditional approaches to deriving hiPSCs require the use of retroviruses or lentiviruses that integrate reprogramming genes into the host genome. Random integration of the reprogramming genes may result in insertional mutagenesis that causes malignant transformation of a clonal cell population[6]. In addition, some of the genes used in the reprogramming process are known proto-oncogenes, and incomplete silencing of these transgenes may result in unknown adverse effects. These challenges have been partially circumvented in mice by the development of non-integrating viral[7], nonviral episomal[8] and excisional[9,10] techniques for reprogramming. Yet clinical translation of these techniques for safer iPSC derivation is challenging because human cells are relatively more resistant to nonviral transfection and are not immediately available in large quantities. Although hiPSCs may be generated by lentiviral transduction with subsequent Cre-*loxP* excision of reprogramming factors[3], residual vector sequences will be left behind in the genome. Transgene-free hiPSCs have been derived from neonatal foreskin fibroblasts using a combination of three episomal plasmids expressing seven reprogramming factors[11]. Alternatively, transgene-free hiPSCs can be derived from fetal or neonatal cells by repeated transduction of proteins in the presence of chemical treatments (e.g., valproic acid)[12]. However, none of the aforementioned techniques for transgene-free hiPSC derivation has been demonstrated with adult donors, a more clinically relevant population. In this study, we describe in detail a protocol for the derivation of transgene-free hiPSCs with a nonviral minicircle DNA reprogramming construct used in conjunction with human adipose stromal cells (hASCs)[13]. This technique is advantageous in translational studies because somatic cells from human adults can be reprogrammed in the absence of genomic modification, viral sequences or proto-oncogenes (such as *c-Myc*), effectively mitigating safety concerns[14]. This protocol can be used to derive hiPSCs from human samples in ~4 weeks using standard molecular biology reagents and cell culture expertise (**Fig. 1**).

Limitations of the protocol

Cells are not transduced with infectious viral particles in this protocol, ensuring a high likelihood of generating transgene-free hiPSCs. However, reprogramming efficiency using this protocol is substantially lower (~0.005%) compared with lentiviral techniques for overexpression of the transcription factors *OCT4*, *SOX2*, *NANOG* and *LIN28A* (referred to hereafter as *LIN28*). Further improvement of reprogramming efficiency may be achieved by treatment with small molecules (e.g., valproic acid)[15] or cell signaling peptides (e.g., Wnt)[16]. Also, users should be aware that the protocol as described here has not yet been successfully applied to the reprogramming of human dermal fibroblasts derived from adult sources. We have found that minicircle-based reprogramming of hASCs as described here is advantageous for deriving transgene-free hiPSCs from adult human donors, a clinically relevant cell source. Such methods offer the ability to develop patient-specific or disease-specific cell lines for exciting new translational and disease modeling studies.

Experimental design

Adipose tissue harvest. hASCs are an attractive source for hiPSC derivation, because they are available in large numbers and are amenable to reprogramming[17,18]. Although skin biopsies are necessarily minimal in size, adipose tissue can be harvested in very large quantities during lipoaspiration procedures, allowing quick

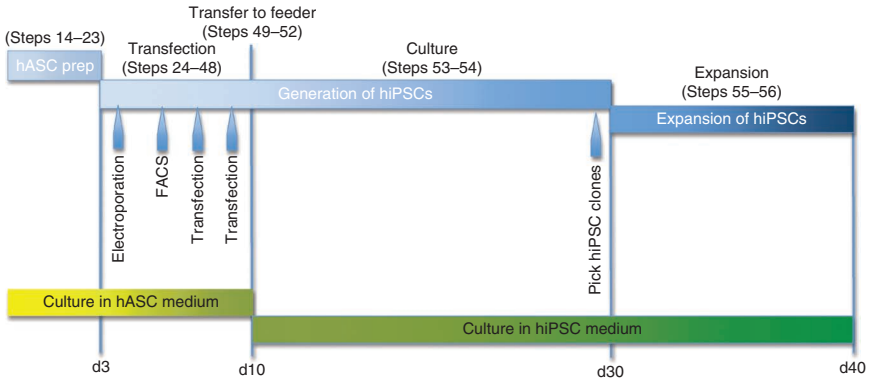

Figure 1 | Schematic of hiPSC derivation protocol. Approximate time frame of the hiPSC derivation process is shown with numbered steps above and cell culture media below.

expansion to a large starting population of hASCs. For example, reprogramming can start as early as 24 h after the liposuction procedure with a large starting cell population of up to 20–30 million cells, whereas 3–4 weeks are required following punch skin biopsy for expansion of the dermal fibroblasts. hASCs are a heterogeneous group of multipotent progenitor cells that can differentiate into adipogenic, osteogenic, chondrogenic and myogenic cell lineages[19,20]. As such, hASCs express relatively high levels of the *c-Myc* and *Klf4* genes, which may underlie the relative ease of their induction to pluripotency. Users should note that the protocol described here has been most successfully applied when using hASCs as the starting cell source, although neonatal fibroblasts (e.g., IMR90) can also be used with lower efficiency.

Minicircle vectors. Minicircle vectors are supercoiled DNA molecules free of bacterial plasmid backbone elements, such as an origin of replication and an antibiotic resistance gene. They primarily consist of a eukaryotic expression cassette, and therefore do not activate exogenous silencing mechanisms to the same extent as plasmids. Therefore, minicircle vectors benefit from higher transfection efficiencies and more stable ectopic transgene expression than plasmid DNA (**Fig. 2**)[21]. The pMC.LGNSO plasmid is the parental DNA construct that is used to produce minicircle reprogramming vector in the initial steps of this protocol (Steps 1–13). Parental plasmid DNA (e.g., pMC.LGNSO) that produces minicircle vectors may be conceptually divided into two parts separated by *attB* and *attP* recognition sequences. Intermolecular recombination between the *attB* and *attP* sequences catalyzed by the ΦC31 integrase (Step 9) yields a minicircle vector separated from the remainder of the plasmid (**Fig. 3**). The minicircle vector contains the reprogramming genes *OCT4*, *SOX2*, *NANOG* and *LIN28*. This expression cassette is isolated and purified from the bacterial suspension (Steps 1–12) before being transfected into somatic cells to induce reprogramming (Step 13–47). The other part of the pMC.LGNSO parental plasmid contains bacterial plasmid backbone elements (e.g., the origin of replication and antibiotic resistance cassette), as well as the ΦC31 integrase and I-SceI restriction enzyme expression cassettes under the control of an L-arabinose-inducible promoter. This part of the plasmid is linearized by I-SceI endonucleolytic cleavage and subsequently degraded (Step 9), allowing isolation of pure minicircle DNA by common plasmid purification procedures (e.g., Qiagen plasmid purification kits). Yield and purity of the minicircle DNA preparation may be optimized by use of plasmids encoding multiple copies of ΦC31 integrase[22], or by the use of *Escherichia coli* strains that encode L-arabinose–inducible I-SceI as part of the bacterial genome (e.g., *E. coli* strain ZYCY10P3S2T). The purified minicircle expression cassette contains the four reprogramming genes plus enhanced green fluorescent protein (EGFP) separated by 2A ribosomal slippage sequences, thereby allowing the equimolar expression of all five proteins from a single RNA transcript (**Fig. 3**). This protocol requires transfection of hASCs with the minicircle preparation three times. First, cells are electroporated to optimize transfection efficiency, then sorted by flow cytometry to enrich for successfully transfected cells, and then finally transfected twice using cationic lipids (e.g., with Lipofectamine) to optimize cell survival.

FACS. Detailed description of the flow cytometric enrichment of EGFP$^+$ hASCs after the first electroporation (Step 37) will vary by institution and is beyond the scope of this protocol. Fortunately, well-established protocols for sorting of EGFP$^+$ cells are readily available[23,24]. For the initial sorting procedure, 5×10^5 untransfected hASCs should be trypsinized and resuspended in fluorescence-activated cell sorting (FACS) buffer alongside the transfected hASCs to set up the gating parameters. Once set, the gating parameters can be saved and untransfected hASCs are no longer needed. We used a FACSAria equipped with FACSDiva software for cell analysis and sorting (http://facs.stanford.edu/). Forward scatter (~82 V) and side

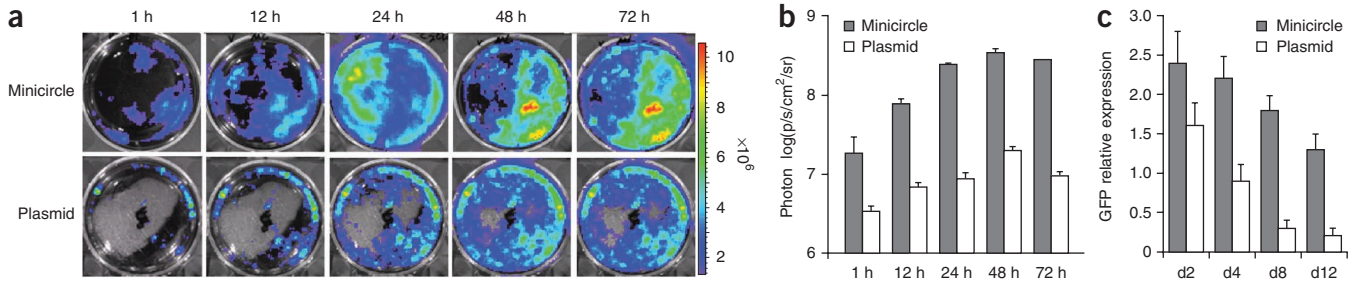

Figure 2 | Minicircle DNA vectors give stronger and more persistent transgene expression than regular plasmids. (**a**) hASCs transfected with either minicircle or regular plasmid carrying identical expression cassettes (cytomegalovirus (CMV) promoter driving an *EGFP*—firefly *luciferase* fusion gene). Representative bioluminescent images are shown of *in vitro* cell cultures at the indicated time points after transfection. (**b**) Quantitative photon counts demonstrate increased luciferase expression in minicircle-transfected cells compared with regular plasmid-transfected cells over 72 h. (**c**) qPCR for *EGFP* shows persistent high expression of the minicircle transgene compared with plasmid over 12 d. For both experiments, the error bars represent the standard deviation from three independent experiments. Reproduced with permission from reference 13.

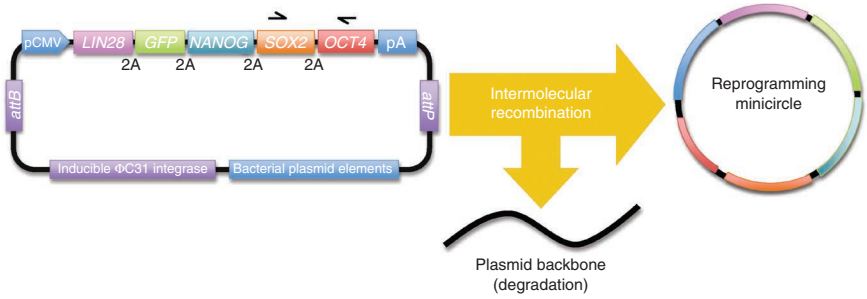

Figure 3 | Minicircle expression vector for hiPSC generation. After induction with L-arabinose, expression of ΦC31 integrase catalyzes intermolecular recombination between the *attB* and *attP* recognition sites, resulting in a minicircle DNA vector separated from plasmid backbone elements. Expression of I-SceI catalyzes linearization of the plasmid backbone, which is subsequently degraded. The minicircle vector contains a CMV promoter (pCMV) driving expression of the *EGFP*, *OCT4*, *SOX2*, *NANOG* and *LIN28* cDNAs separated by 2A ribosomal slippage sequences. The SV40 polyA (pA) site ensures transcription termination, polyadenylation and efficient expression of the transcript. Arrows above the minicircle vector show primers 2 and 5 (**Table 1**); these are used for screening of the vector's integration into genomic DNA.

scatter (~176 V) were adjusted to exclude cell aggregates and debris. The band-pass filter for fluorescein isothiocyanate detection was used (~537 V), and cells were analyzed at a flow rate between 200 and 1,000 events per second in a stream formed from a nozzle with a 70-µm orifice.

Picking colonies. hiPSC-like colonies with a tightly packed, dome-like structure first appear at approximately day 18 (Steps 53 and 54; **Fig. 4**). Colonies become large enough to be manually picked up and transferred to a separate irradiated mouse embryonic fibroblast (iMEF) feeder layer at approximately day 28 (Steps 55 and 56). At early stages, colonies remain EGFP$^+$, although with continued culture and passaging, *bona fide* hiPSC clones will become EGFP$^-$.

Screening of the resulting hiPSC lines. Expression of the minicircle-derived reprogramming cassette can be easily monitored throughout the procedure by EGFP fluorescence. At the conclusion of the procedure, however, *bona fide* hiPSC colonies will no longer be dependent on exogenous reprogramming factor expression, and will thereby be EGFP negative. The pluripotency of the derived hiPSC lines can be verified using standard assays described in detail elsewhere, including reverse transcriptase PCR (RT-PCR) and immunocytochemistry for pluripotent markers, as well as embryoid body differentiation and teratoma formation[25]. In addition to these standard protocols, the resulting hiPSCs can be screened for rare integration events by a simple PCR-based assay for the presence of the transgene in genomic DNA. The minicircle-derived reprogramming cassette contains the *LIN28*, *NANOG*, *SOX2* and *OCT4* cDNA sequences in succession (**Fig. 3**), whereas endogenous genomic DNA loci expressing these genes contain introns and are present on disparate chromosomes. Therefore, PCR of extracted genomic DNA using primers annealing to two contiguous minicircle reprogramming genes (e.g., *SOX2* and *OCT4*) can readily identify rare integration events of the transgene into genomic DNA (Steps 57–60).

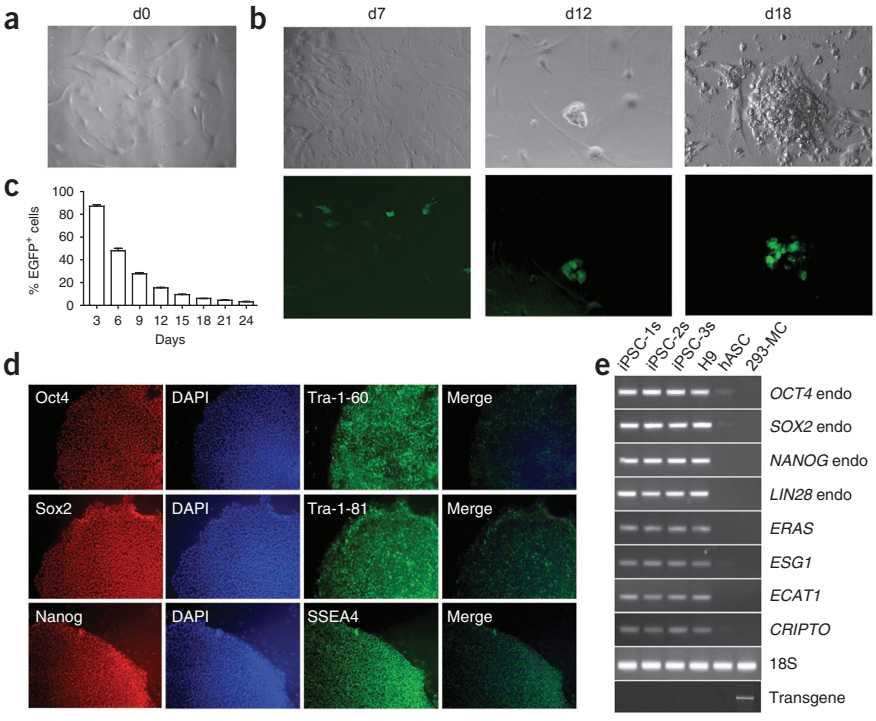

Figure 4 | Characterization of developing and *bona fide* hiPSCs. (**a**) Bright-field image of untransfected hASCs. (**b**) Bright-field (upper) and fluorescence (lower) images of reprogramming hASCs at days 7, 12 and 18 post-transfection. (**c**) Flow cytometric analysis of sorted hASCs after transfection with the minicircle vector on day 0. Percentage of EGFP$^+$ cells is plotted against days post-transfection. (**d**) Representative images of minicircle-derived hiPSCs immunostained for pluripotency markers as described in Steps 52–61. Nuclear antigens requiring permeabilization, such as Oct4, Nanog and Sox2, were stained using Alexa Fluor 546–conjugated secondary antibody (red emission), while cell surface antigens Tra-1-60, Tra-1-81 and SSEA4 were stained using Alexa Fluor 488–conjugated secondary antibody (green emission). Merged images (far right) show localization of Tra-1-60, Tra-1-81 and SSEA4 antigens (green) to the cell surface in contrast to the nuclear DAPI stain (blue). (**e**) qPCR analysis of three undifferentiated minicircle-derived hiPSC lines compared with H9 hESCs, hASCs and 293-MC negative control (HEK 293 cells 24 h after transfection with the minicircle vector). Robust expression of pluripotency markers including endogenous (endo) OCT4, SOX2, NANOG, LIN28, *ERAS*, *TLE1* (also known as *ESG1*), *C6ORF221* (also known as *ECAT1*) and *TDGF1* (also known as *CRIPTO*) was observed in the hiPSC and hESC lines, with 18S rRNA serving as a positive control. Genomic DNA was also isolated to screen for integration of the minicircle vector using primers specific for the *Oct4-Sox2* transgene (bottom). Reproduced with permission from reference 13.

MATERIALS

REAGENTS

- pMC.LGNSO plasmid (available on request or from StemCell Technologies, cat. no. 05820)
- ZYCY10P3S2T *E. coli* strain (available on request) or TOP10 competent cells (Invitrogen, cat. no. 4040) ▲ **CRITICAL** Use these designated *E. coli* strains to achieve optimal yield of minicircle DNA.
- Terrific Broth (TB) powder (Invitrogen, cat. no. 22711-022)
- Luria-Bertani (LB) broth powder (Invitrogen, cat. no. 12780-052)
- LB-agar plates for kanamycin-resistant *E. coli* strains (Invitrogen, cat. no. 45-0043)
- 1N NaOH solution (Agilent Technologies, cat. no. 5062-8576) ! **CAUTION** This solution causes burns, is harmful when swallowed, and is toxic when in contact with skin and eyes; use protective gloves and safety glasses when handling.
- L-arabinose (Sigma, cat. no. A3256)
- Kanamycin sulfate solution 10 mg ml^{-1} (Invitrogen, cat. no. 15160)
- Opti-MEM I reduced-serum medium (Invitrogen, Gibco, cat. no. 31985)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- DMEM, high glucose with L-glutamine (Invitrogen, Gibco, cat. no. 11965)
- FBS (Invitrogen, Gibco, cat. no. 10437)
- FBS, ES-Cell (Invitrogen, Gibco, cat. no. 16141)
- Penicillin-streptomycin (Invitrogen, Gibco, cat. no. 15070)
- Nonessential amino acids (Invitrogen, cat. no. 11140-050)
- 2-Mercaptoethanol (Invitrogen, cat. no. 21985-023) ! **CAUTION** Flammable; harmful if swallowed; toxic when in contact with skin and eye; use protective gloves and safety glasses when handling.
- Gelatin (Sigma, cat. no. G1890)
- Knockout DMEM (Invitrogen, Gibco, cat. no. 10829)
- PBS, without Ca^{2+} and Mg^{2+} (Invitrogen, Gibco, cat. no. 10010)
- Hank's balanced salt solution (HBSS, Invitrogen, cat. no. 14025)
- FACS Buffer, PBS-based (Gibco, cat. no. 13150)
- Bovine serum albumin (Sigma, cat. no. A9418)
- Triton X-100 (Sigma, cat. no. T8787)
- Antibiotic/antimycotic solution, 100× (Mediatech, cat. no. 30-004-CI)
- Type II collagenase (Invitrogen, cat. no. 17101-015)
- Irradiated mouse embryonic fibroblast (iMEF) feeder cells from CF1 mice (GlobalStem, cat. no. GSC-6001G)
- Restriction endonuclease HindIII, SphI, NdeI or XhoI (New England Biolabs)
- BlueJuice 10× DNA loading dye (Invitrogen, cat. no. 10816-015)
- TrackIt 1kb+ DNA ladder (Invitrogen, cat. no. 10488-085)
- HiSpeed plasmid maxi kit (Qiagen, cat. no. 12662)
- ArchivePure DNA cell/tissue kit (5 Prime, cat. no. 2300810)
- Nucleofector kit R (Lonza, cat. no. VCA-1001)
- Accuprime high fidelity Taq polymerase (Invitrogen, cat. no. 12346-086)
- ES cell marker characterization kit (Millipore, cat. no. SCR002)
- Formaldehyde solution (Sigma, cat. no. F8775)
- RNeasy mini kit (Qiagen, cat. no. 74104)
- iScript cDNA synthesis kit (Bio-Rad, cat. no. 170-8890)
- GoTaq qPCR master mix (Promega, cat. no. A6001)
- TrypLE Express (Invitrogen, cat. no. 12605-028)
- Basic fibroblast growth factor (bFGF) (Chemicon, cat. no. GF003)
- Povidone-iodine (Betadine)
- Alexa Fluor 488 or 546 dye
- 4,6-diamidino-2-phenylindole (DAPI)
- Distilled, deionized water (ddH2O)
- Nuclease-free water

EQUIPMENT

- Cell culture incubator, 95% air and 5% CO_2, humidified
- Cell culture hood
- Automated cell counter (e.g., Countess, Invitrogen)
- Inverted epifluorescence microscope
- Fluorescence-activated cell sorter (FACSAria, BD Biosciences)
- 5-ml round-bottom tube (BD Falcon, cat. no. 352063)
- 1.5-ml microcentrifuge tubes
- pH meter
- Cuvette spectrophotometer (e.g., Eppendorf BioPhotometer)
- 2-Liter Erlenmeyer glass flask
- 150-mm tissue culture dish (Falcon, cat. no. 353025)
- 100-mm tissue culture dish (Falcon, cat. no. 353003)
- 6-well tissue culture dish (Falcon, cat. no. 353046)
- 15-ml conical tube (Falcon, cat. no. 352196)
- 50-ml conical tube (Falcon, cat. no. 352070)
- 100-µm cell strainer (Falcon, cat. no. 352360)
- Nucleofector device (Lonza) ▲ **CRITICAL** The parameters of the nucleofection programs are proprietary. We have been unable to obtain high transfection efficiencies using other electroporation systems (e.g., Bio-Rad GenePulser).
- Dissecting forceps. ! **CAUTION** Sterilize by autoclave.
- StepOnePlus real-time PCR system (Applied Biosystems, cat. no. 4376600)
- Vacuum filter system, 0.44 µm

REAGENT SETUP

Minicircle induction broth First, prepare a stock of 20% (wt/vol) L-arabinose by dissolving 2 g L-arabinose in 100 ml of ddH_2O. Stock solution can be frozen at −20 °C for up to 6 months. To prepare induction broth, mix 384 ml LB, 16 ml 1N NaOH and 0.4 ml 20% (wt/vol) L-arabinose (final concentration of L-arabinose is 0.01%). Make fresh just before use.

hASC medium Combine high-glucose DMEM with L-glutamine containing 10% (vol/vol) FBS, 0.5% (vol/vol) penicillin-streptomycin (final concentration 50 units ml^{-1} penicillin and 50 µg ml^{-1} streptomycin). Store at 4 °C for ~1 month.

hiPSC medium Combine knockout DMEM, 20% (vol/vol) ES cell FBS, 0.1 mM nonessential amino acids, and 0.1 mM 2-mercaptoethanol and bFGF to a final concentration of 8 ng ml^{-1}. Store at 4 °C for ~2 weeks.

0.075% Type II collagenase Add 75 mg of type II collagenase to 100 ml of ddH_2O (0.075% (wt/vol)) and filter-sterilize using a 0.44-µm vacuum-driven filter system. Make 10-ml aliquots of solution and store them at −20 °C for up to 2 months.

Gelatin solution Dissolve 0.5 g gelatin powder in 500 ml of distilled water; autoclave and store at 4 °C for up to 2 months.

Gelatin-coated culture dishes Add a sufficient volume of gelatin solution to cover the surface of the plate or well. Incubate the dish for at least 30 min at 37 °C. Before use, aspirate excess gelatin solution.

PROCEDURE

Minicircle DNA production ● TIMING 3 d

1| Verify the identity and integrity of the pMC.LGNSO plasmid by restriction digestion. Incubate a mixture of the following reagents in a 1.5-ml microcentrifuge tube at 37 °C for >1 h:

pMC.LGNSO plasmid	1 µg
NEB buffer	2 µl
10× BSA	2 µl
Restriction endonuclease (HindIII/SphI/NdeI/XhoI)	0.5 µl
ddH_2O	up to 20 µl

2| Add 2 µl of 10× loading dye to the restriction digestion mixture. Load the entire mixture into the well of a 0.8% (wt/vol) agarose gel. Load ~5 µl of a DNA ladder of choice (e.g., TrackIt 1kb+) into a neighboring well. Electrophorese at a constant voltage of 100 V for 70 min. Verify that the following double-cutters produce restriction fragment lengths as shown below. If further confirmation of the identity of the plasmid is desired, it can be sequenced using primer 17 (**Table 1**).

Restriction endonuclease	Fragment size (bp)
HindIII	2,091; 2,659
SphI	1,800; 3,950
NdeI	1,030; 4,720
XhoI	369; 5,381

3| Begin transforming the *E. coli* strain (ZYCY10P3S2 or TOP10) with pMC.LGNSO plasmid by adding 100 pg to 50 ng of plasmid to a 50-µl aliquot containing 1 × 10^9 colony-forming units (c.f.u.) of chemically competent *E. coli*. Place on ice for 10 min.

4| Heat-shock in 42 °C water bath for 30 s. Next, place on ice for 2 min.

5| Spread transformed *E. coli* on a prewarmed LB-agar kanamycin-selective plate. Incubate overnight 12–16 h at 37 °C.

6| The next day, inoculate 5 ml of LB broth containing kanamycin (50 µg ml^{-1}) with a colony from the freshly streaked plate. Incubate this starter culture at 37 °C with agitation at 250 r.p.m. for 8 h.

7| Autoclave 400 ml of TB in a 2-liter Erlenmeyer flask. When flask is cool enough to touch (~55 °C), add 0.2 ml of 10 mg ml^{-1} kanamycin sulfate solution to a final concentration of 50 µg ml^{-1}.

8| Inoculate 100 µl of the starter culture from Step 5 into 400 ml of TB prepared in Step 6 and incubate overnight for 16–18 h at 37 °C with shaking at 250 r.p.m.

9| The next day, take a 0.5-ml sample of the overnight bacterial culture and confirm that the optical density reading at 600 nm (OD_{600}) is between 4 and 5, and that the pH is 6.5.

10| Combine 400 ml of the minicircle induction broth (see REAGENT SETUP) with 400 ml of the overnight culture into a 2-liter Erlenmeyer flask. Incubate at 32 °C with shaking at 250 r.p.m. for >5 h.
▲ **CRITICAL STEP** Longer incubation periods of up to 9 h will yield minicircle DNA of greater purity.

11| Pellet bacterial cells by centrifugation at 6,000*g* for 15 min at 4 °C.

12| Discard supernatant and resuspend the bacterial pellet in 100 ml of Qiagen Buffer P1 (Qiagen Buffers P1, P2, P3 and EB are included in the HiSpeed maxi prep kit). Follow the maxi prep kit protocol per the manufacturer's instructions, but use double the volume of buffers P1, P2 and P3 (100 ml each). Elute DNA using 200 µl of Buffer EB or Tris-EDTA buffer (pH 8).

13| Measure DNA concentration and purity using a spectrophotometer. Minicircle DNA is now ready for subsequent transfection. For further confirmation of the identity and integrity of minicircle DNA preparation, dilute a sample (0.5 µg) in an appropriate volume of 10× DNA loading dye and electrophorese on a 0.8% (wt/vol) agarose gel at 100 V for 70 min alongside a DNA ladder. Verify that a clean, solitary 5.6-kb band is present with no smaller bands indicating nicked or degraded DNA.
■ **PAUSE POINT** Minicircle DNA preparations can be frozen in 5-µg aliquots at −20 °C indefinitely. Avoid repeated freeze-thaw cycles.

Derivation of hASCs ● TIMING 3 d (days 1–3)
14| Obtain an adipose tissue sample by liposuction aspiration.
! **CAUTION** Adipose tissue should be obtained by a surgeon or other appropriately trained physician. Informed consent should be obtained from the individual in accordance with institutional and international patient protection guidelines.

TABLE 1 | Primer sequences.

#	Name	Direction	Sequence	Size (bp)
1	*OCT4*	352F	5′-ACCCCTGGTGCCGTGAA-3′	189
2		541R	5′-GGCTGAATACCTTCCCAAATA-3′	
3	*OCT4* endo	912F	5′-GCGATCAAGCAGCGACTA-3′	399
4		1311R	5′-TTCACCTTCCCTCCAACC-3′	
5	*SOX2*	890F	5′-CAGCGCATGGACAGTTAC-3′	320
6		1210R	5′-GGAGTGGGAGGAAGAGGT-3′	
7	*SOX2* endo	1183F	5′-CCCTGTGGTTACCTCTTCC-3′	258
8		1441R	5′-CTCCCATTTCCCTCGTTT-3′	
9	*NANOG*	433F	5′-AAAGGCAAACAACCCACT-3′	269
10		702R	5′-GCTATTCTTCGGCCAGTT-3′	
11	*NANOG* endo	1197F	5′-CTCCTCCCATCCCTCATA-3′	105
12		1302R	5′-AGGCTCCAACCATACTCC-3′	
13	*LIN28*	270F	5′-GTTCGGCTTCCTGTCCAT-3′	121
14		391R	5′-CTGCCTCACCCTCCTTCA-3′	
15	*LIN28* endo	1135F	5′-AGCCAAGCCACTACATTC-3′	299
16		1434R	5′-AGATACGTCATTCGCACA-3′	
17	CMV seq	F	5′-AAGCAGAGCTGGTTTAGTGAA-3′	

Primers 1–16 may be used for qPCR analysis of resulting hiPSC clones. Primer pairs specific for the endogenous transcript (3 and 4, 7 and 8, 11 and 12, 15 and 16) anneal to the untranslated region (UTR) of the transcript, which is absent on the minicircle-derived transcript. Used in conjunction, primers 2 and 5 amplify a specific 1.1-kb region that is unique to the minicircle construct as shown in **Figure 3**. These two primers can be used to screen genomic DNA for integration of the minicircle-derived transgene as described (Steps 62–65). Primer 17 anneals to the CMV promoter transcriptional start site and may be used for sequence verification of the pMC.LGNSO plasmid.

15| Immediately place the adipose tissue specimen on ice.

16| Prepare four 50-ml conical tubes containing 25 ml of serial dilutions of povidone-iodine (Betadine) and PBS in ratios of 1:0 (undiluted Betadine), 1:1, 1:2 and 1:5. Wash the specimen by immersing it sequentially in each dilution for 5 s each.

17| Wash the specimen twice by immersion in 25 ml PBS in two separate 50-ml conical tubes.

18| Place the specimen in a fresh 50-ml conical tube and measure the volume of the tube occupied by adipose tissue (~10 to 15 ml). Digest the tissue by adding an equal volume of 0.075% (wt/vol) Type II collagenase (see REAGENT SETUP). Incubate the digestion mixture in a 37 °C water bath with agitation at 125 r.p.m. for 30 min.

19| Inactivate the collagenase by adding an equal volume of serum-containing hASC growth medium (see REAGENT SETUP).

20| Pellet the stromal vascular fraction containing hASCs by centrifugation at 1,200*g* for 5 min.

21| Aspirate the supernatant. Resuspend the pellet in 5 ml of hASC medium. Count the cells using an automated cell counter to verify that cell yield is ~1 to 5 × 10^6 cells.

22| Filter the resuspended cells through a 100-µm cell strainer into a 15-ml conical tube.

23| Add hASC medium to a total volume of 15 ml. Seed the entire 15-ml hASC cell suspension (~1 to 5 × 10^6 cells) on a 150-mm tissue culture dish for further expansion in a humidified incubator at 37 °C, 5% CO_2.

▲ CRITICAL STEP hASCs can be passaged and expanded using standard cell culture techniques[26]. Our laboratory uses TrypLE Express reagent for digestion and split ratios of 1:4 every 3–5 d. hASCs may be placed in a cryopreservation solution (10% DMSO, 90% FBS) in aliquots of 2×10^5 cells per cryovial and immersed in liquid nitrogen indefinitely.
▲ CRITICAL STEP For optimal hiPSC generation efficiency, electroporate hASCs within three passages to minimize replicative senescence.

Induction of pluripotency: hASC transfection 1 ● TIMING 3 d (days 4–6)
24| Aspirate hASC medium from the 150-mm dish containing hASCs at ~70% confluency. Add 10 ml PBS and rock the plate to wash. Aspirate the PBS.

25| Add 2 ml TrypLE Express. Incubate at 37 °C for 2–5 min until cells round up and detach.

26| Add 4 ml of hASC growth medium to stop enzymatic digestion. Pipette the cell suspension up and down to wash all hASCs off the plate. Pipette the cell suspension into a 15-ml conical tube. Wash the 150-mm dish with another 4 ml of hASC medium and add it to the 15-ml conical tube. Count cells using an automated cell counter.

27| Pellet $1–2 \times 10^6$ hASCs by centrifugation at 200*g* for 5 min.

28| Aspirate the supernatant completely. Gently resuspend the hASC pellet in 100 µl of Nucleofector Solution R.
▲ CRITICAL STEP Ensure that Nucleofector Solution R is at room temperature (25 °C). Ensure that supplement has been added to the solution per the manufacturer's instructions.

29| Add 5 µg of minicircle DNA from Step 13 to the cell suspension.

30| Gently transfer the cell-DNA suspension to a cuvette using a micropipette. Close the cuvette with the cap. Place the cuvette in the Lonza Nucleofector device and execute program 'U-023'.

31| Gently remove the cuvette from the device after completion of the program.

32| Add ~500 µl of hASC medium (preferably prewarmed to 37 °C) to the cuvette using the plastic pipette. Next, use the same plastic pipette to gently transfer the cell suspension to a 150-mm dish. Avoid repeated aspiration of the sample. Carefully distribute the cells on the 150-mm dish by extremely gentle rocking. Place in 37 °C incubator for expansion.

33| The next day, aspirate hASC medium containing non-viable cells. Add fresh hASC growth medium. Place 150-mm dish in 37 °C incubator overnight.

34| The next day, aspirate hASC medium. Wash with PBS.

35| Add 2 ml of TrypLE Express. Incubate at 37 °C for 2–5 min until cells round up and detach from the plate.

36| Add 4 ml of hASC medium. Pipette the cell suspension up and down to wash all hASCs off the plate. Collect the cell suspension into a 15-ml conical tube. Pellet cells by centrifugation at 200*g* for 5 min. Aspirate supernatant. Resuspend cell pellet in 1 ml of FACS buffer supplemented with 2% (vol/vol) FBS.

37| Isolate 1×10^5 $EGFP^+$ cells by flow cytometry[23,24]. Set the sorting gate specifically for green fluorescence with a 488-nm laser for EGFP, and maximize yield of $EGFP^+$ cells (~2% to 10% of the population). Pellet sorted cells by centrifugation at 1,200*g* for 5 min. Aspirate FACS buffer supernatant. Resuspend $EGFP^+$ hASC pellet in 4 ml hASC medium. Split cells into two wells of a six-well tissue culture plate (2 ml per well). Place in 37 °C incubator overnight.
▲ CRITICAL STEP Return hASCs to a humidified 37 °C incubator as quickly as possible to maximize cell viability. Be sure to have added penicillin-streptomycin antibiotic to medium to reduce the risk of contamination after the cell sorting procedure.
? TROUBLESHOOTING

Induction of pluripotency: hASC transfection 2 ● TIMING 2 d (days 7–8)
38| Dilute 10 µg of minicircle DNA from Step 13 in 500 µl of Opti-MEM in a 1.5-ml microcentrifuge tube.

39| Add 20 µl of Lipofectamine 2000 to 500 µl of Opti-MEM in a separate 1.5-ml microcentrifuge tube. Incubate at room temperature for 5 min.

40| Add the Lipofectamine-containing solution to the DNA-containing solution gently and incubate at room temperature for 15–20 min.

41| Aspirate hASC medium from the six-well plate containing hASCs (from Step 37). Add 2 ml PBS per well and rock the plate back and forth to wash. Aspirate the PBS. Next, add 1 ml of fresh hASC medium per well.

42| Add 500 µl of the Lipofectamine-DNA solution to each well. Medium does not need to be refreshed, but if desired, wait until at least 4 h have elapsed since the addition of the Lipofectamine-DNA solution before refreshing the medium. Incubate overnight in a humidified incubator at 37 °C.

43| The next day, aspirate medium from the wells containing hASCs. Replace with 2 ml of fresh hASC medium. Return to the incubator overnight.

Induction of pluripotency: hASC transfection 3 ● TIMING 1 d (day 9)

44| Repeat Steps 38–43. **Figure 4b** shows representative images of reprogramming hASCs during the transfection procedure. ~20% of the hASCs will remain $EGFP^+$ by day 10 of the procedure (**Fig. 4c**).

45| The next day, thaw one vial containing 5×10^6 cryopreserved iMEFs (irradiated MEFs, isolated from CF1 mice) by gently swirling in 37 °C water bath until most, but not all, of the contents have thawed.

46| Transfer 10 ml of hASC medium to an empty 15-ml conical tube. Slowly add iMEF cell suspension to the conical tube using a micropipettor.

47| Centrifuge iMEF cell suspension at 200*g* for 5 min at room temperature. Aspirate supernatant.

48| Resuspend cells in 1 ml of hASC medium, then count cells using an automated cell counter (or hemacytometer). Add hASC medium to adjust the cell concentration to 2×10^6 cells per ml. Plate 1×10^6 cells per 100-mm gelatin-coated dish. 2×10^6 cells is sufficient to prepare 2 × 6-well plates or 2 × 10-cm dishes or 2 × 12-well plates or 16 × 4-well plates (~120 cm^2). Place in a humidified incubator at 37 °C overnight.

Induction of pluripotency: transfer to iMEF feeder layer ● TIMING 1 d (day 10)

49| The next day, aspirate hASC medium from the six-well plate containing hASCs from Step 43. Add 2 ml of PBS per well, wash by rocking back and forth, and aspirate PBS. Add 500 µl of TrypLE Express per well and incubate at 37 °C for 2–5 min until cells round up and detach.

50| Aspirate hASC medium from the 100-mm gelatin-coated dish containing iMEFs. Add 6 ml of PBS, wash by rocking back and forth, and aspirate PBS. Add 8 ml of hiPSC medium to the dish.

51| Add 500 µl of hiPSC medium to each well of the six-well plate to stop enzymatic digestion. Gently pipette the solution over the surface of the well 5 or 6 times to detach all hASCs.

52| Transfer the hASC cell suspension from the six-well plate to the 100-mm dish containing the iMEF feeder layer. Rock the cell suspension in several quick back-and-forth motions, then carefully place the 100-mm dish in the humidified incubator at 37 °C overnight.

? TROUBLESHOOTING

Culturing reprogramming hASCs on iMEF feeder layer ● TIMING 20 d (days 11–30)

53| Aspirate and replace the 100-mm dish with 10 ml of fresh hiPSC medium every 24 h.

54| Between days 23 and 30, colonies with morphologies similar to hESCs will begin to appear.

Picking and expanding hiPSC colonies ● TIMING 10 d (days 30–40)

55| Track hiPSC-like colonies showing a mosaic green fluorescence pattern under an epifluorescence microscope daily (**Fig. 4**). Surrounding unreprogrammed or partially reprogrammed hASCs may appear $EGFP^+$. Between 5 and 15 hiPSC colonies are expected from 2×10^5 $EGFP^+$ hASCs isolated during FACS (Step 37).

56| When mosaic EGFP colonies reach a size of 0.5–1.0 mm in diameter, they can be cut into small clumps using a pulled Pasteur pipette. Subsequently, they can be picked up using a micropipettor set at 2 µl and then transferred to the wells of a 6-well plate containing fresh iMEFs for subcloning culture. This subcloning procedure may be repeated until $EGFP^+$ cells are completely absent. hiPSC clones can then be cultured and expanded according to standard hESC/hiPSC culture techniques[27,28]. **? TROUBLESHOOTING**

Screening of resulting hiPSC clones for genomic integration ● TIMING 1 d

57| Isolate genomic DNA from one well of a 50–70% confluent six-well plate of hiPSCs with the ArchivePure DNA extraction kit, following the manufacturer's instructions. Also isolate total DNA from a cell line (e.g., HEK 293 cells) that has been transfected with the minicircle reprogramming cassette as a positive control.

■ **PAUSE POINT** DNA preparations can be frozen at 4 °C for 3 months or −20 °C indefinitely. Avoid repeated freeze-thaw cycles.

58| Add the following components to a 0.2-ml PCR tube:

Genomic DNA	500 ng
10 µM primer 2 (**Table 1**; *OCT4* reverse primer)	1 µl
10 µM primer 5 (**Table 1**; *SOX2* forward primer)	1 µl
Accuprime buffer II	5 µl
Accuprime Taq	0.2 µl
ddH_2O	up to 50 µl

59| Run the PCR reactions in a thermal cycler programmed with the following settings:

94 °C for 3 min
35 cycles of: 94 °C for 15 s, 60 °C for 30 s, 68 °C for 1 min
68 °C for 7 min
Hold at 4 °C

60| Perform electrophoresis with 25 µl of each of the above reactions on a 1% (wt/vol) agarose gel. Fragment size for the integrated minicircle transgene is 1,102 bp. Select clones that do not contain a band for the integrated minicircle plasmid for further culture. Any clones containing the 1,102-bp PCR product may have integrated the reprogramming factor transgenes.

Characterization of hiPSC clones: RT-PCR ● TIMING 1 d

61| Extract total RNA from one well of 50–70% confluent six-well plate containing established hiPSC clones; use the Qiagen RNeasy mini kit following the manufacturer's instructions.

62| Synthesize cDNA with the iScript cDNA synthesis kit according to the manufacturer's instructions.

63| Perform quantitative PCR (qPCR) with cDNA template as follows:

2× PCR Master Mix	25 µl
10 µM forward primer (see **Table 1**)	1 µl
10 µM reverse primer (see **Table 1**)	1 µl
cDNA template	50–500 ng
Nuclease-free H_2O	up to 50 µl

64| Run qPCRs in a thermal cycler or real-time PCR system according to the following parameters (using SYBR Green settings):

95 °C for 2 min
30 cycles of: 95 °C for 15 s, 60 °C for 1 min
Hold at 4 °C

65| Analyze cycle threshold (C_T) values for gene expression fold change using the $\Delta\Delta C_T$ method[29] or perform gel electrophoresis of PCR products on a 1% (wt/vol) agarose gel (**Fig. 4**).

Characterization of hiPSC clones: immunocytochemistry ● TIMING 1 d

66| Aspirate the medium from one well of 50–70% confluent six-well plate of established hiPSCs.

67| Wash the cells with 2 ml of PBS.

68| Add 2 ml of PBS containing 10% formalin and fix the cells by incubation at room temperature for 10 min.

69| Wash the cells once with 2 ml of PBS.

70| Aspirate the PBS and add 1 ml of PBS containing 1% (wt/vol) bovine serum albumin and 0.1% (vol/vol) Triton X-100. Incubate 45 min at room temperature.
▲ **CRITICAL STEP** Exclude Triton X-100 if staining cell surface markers, such as Tra-1-60, Tra-1-81 or SSEA-4. Triton X-100 is only necessary for staining of intracellular antigens such as OCT4, NANOG and SOX2.

71| Aspirate the blocking solution and add 1 ml of the primary antibody diluted to the appropriate concentration. Incubate overnight at 4 °C.

72| Wash the cells three times for 5 min each with PBS.

73| Add 1 ml of secondary antibody conjugated to Alexa Fluor 488 or 546 supplemented with 4,6-diamidino-2-phenylindole (DAPI) at 1 μg ml^{-1} and incubate for 45 min in the dark.

74| Wash secondary antibody twice with 2 ml of PBS.

75| Observe the cells with a fluorescent microscope equipped with the appropriate filters.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
37	Low yield of GFP^+ cells	Low transfection efficiency	Use high-quality DNA preparations. Consider using an endotoxin removal kit to further purify the minicircle DNA preparation of contaminants that may limit efficient transfection
			Begin with higher numbers of starting cell source; up to 1×10^7 cells can be used during the initial transfection
52	Cells do not attach	Trypsin-damaged cells	Do not incubate hASCs in TrypLE Express for longer than 5 min. Cells can be gently scraped from the surface of the plate to aid detachment
		Cells experience excessive shear force	Pipette gently
56	Unstable hiPSC clones	Plasmid integration into genome	Use high-quality DNA preparations. Store DNA at −20 °C and avoid repeated freeze-thaw cycles as this may cause strand nicking and degradation
		Partial reprogramming	Choose high-quality clones. Colonies with good hESC morphology are tightly compacted with sharp borders and they contain cells with prominent, large, round nucleoli and scant cytoplasm. Use freshly prepared medium and ensure that the appropriate amount of bFGF has been added

● TIMING

Step 1–13, Preparation of minicircle DNA: 3 d
Step 14–23, Derivation of hASCs: 3 d
Step 24–48, Induction of pluripotency: 7 d
Step 49–52, Transfer to iMEF layer: 1 h
Steps 53 and 54, Culturing reprogramming hASCs on iMEF feeder layer: 20 d
Steps 55 and 56, Picking and expanding hiPSC colonies: 10 d
Steps 57–60, Screening of resulting hiPSC clones: 1 d
Steps 61–65, Characterization of hiPSC clones: RT-PCR: 1 d
Steps 66–75, Characterization of hiPSC clones: immunocytochemistry: 1 d

ANTICIPATED RESULTS

Transfection efficiency can be monitored by EGFP fluorescence under an inverted epifluorescence microscope or by flow cytometry. During the plasmid transfections, floating cells will appear because of the cytotoxicity of electroporation and Lipofectamine. The initial nucleofection procedure has a transfection efficiency of 2–10%, and EGFP fluorescence of the sorted population declines over time as shown in **Figure 4c**. Picked hiPSC clones will stain positively for pluripotency markers (Tra-1-60, SSEA-4), express high transcript levels of pluripotency genes (*OCT4*, *SOX2*, *NANOG* and *TGFI* (also known as *CRIPTO*)), and display hESC-like morphology, such as large, round nucleoli and scant cytoplasm (**Fig. 4**). We have successfully derived 22 hiPSC lines using this protocol from three adult donors. We calculate our reprogramming efficiency using this technique as ~0.005%, although any such calculation may be obviated by the splitting of cells during the procedure.

ACKNOWLEDGMENTS We are grateful to N. Sun for expert assistance with cell culture techniques. We thank Z.Y. Cheng for help with minicircle production techniques. We acknowledge funding support from Howard Hughes Medical Institute (K.H.N.); Mallinckrodt Foundation, National Institutes of Health (NIH) DP2OD004437, RC1AG036142, Burroughs Wellcome Foundation, and the American Heart Association 0970394N (J.C.W.); NIH R90DK07010301, NIH R21DE018727, NIH R21DE019274, NIH RC2DE020771, the Oak Foundation and the Hagey Laboratory for Pediatric Regenerative Medicine (M.T.L.); NIH RC1HL100490-02 (J.C.W. and M.T.L.); and NIH U01HL099776 (R.C.R.).

AUTHOR CONTRIBUTIONS K.H.N., F.J. and J.C.W. prepared most of the paper. R.C.R., M.A.K. and M.T.L. provided advice and proofread the paper.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Published online at http://www.natureprotocols.com/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872 (2007).
2. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920 (2007).
3. Soldner, F. *et al.* Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* **136**, 964–977 (2009).
4. Dimos, J.T. *et al.* Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**, 1218–1221 (2008).
5. Lee, G. *et al.* Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* **461**, 402–406 (2009).
6. Hacein-Bey-Abina, S. *et al.* LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**, 415–419 (2003).
7. Stadtfeld, M. *et al.* Induced pluripotent stem cells generated without viral integration. *Science* **322**, 945–949 (2008).
8. Okita, K. *et al.* Generation of mouse induced pluripotent stem cells without viral vectors. *Science* **322**, 949–953 (2008).
9. Kaji, K. *et al.* Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* **458**, 771–775 (2009).
10. Woltjen, K. *et al.* piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* **458**, 766–770 (2009).
11. Yu, J. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324**, 797–801 (2009).
12. Zhou, H. *et al.* Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* **4**, 381–384 (2009).
13. Jia, F. *et al.* A nonviral minicircle vector for deriving human iPS cells. *Nat. Meth.* **7**, 197–199 (2010).
14. Miura, K. *et al.* Variation in the safety of induced pluripotent stem cell lines. *Nat. Biotech.* **27**, 743–745 (2009).
15. Huangfu, D. *et al.* Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat. Biotech.* **26**, 795–797 (2008).
16. Marson, A. *et al.* Wnt signaling promotes reprogramming of somatic cells to pluripotency. *Cell Stem Cell* **3**, 132–135 (2008).
17. Sun, N. *et al.* Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc. Natl. Acad. Sci. USA* **106**, 15720–15725 (2009).
18. Aoki, T. *et al.* Generation of induced pluripotent stem cells from human adipose-derived stem cells without c-MYC. *Tissue Eng. Part A* **6**, 6 (2010).
19. Guilak, F. *et al.* Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J. Cell Physiol.* **206**, 229–237 (2006).
20. Bunnell, B.A. *et al.* Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **45**, 115–120 (2008).
21. Chen, Z.-Y. *et al.* Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression *in vivo*. *Mol. Ther.* **8**, 495–500 (2003).
22. Chen, Z.Y., He, C.Y. & Kay, M.A. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression *in vivo*. *Hum. Gene Ther.* **16**, 126–131 (2005).
23. Galbraith, D.W., Anderson, M.T. & Herzenberg, L.A. Flow cytometric analysis and FACS sorting of cells based on GFP accumulation. *Methods Cell Biol.* **58**, 315–341 (1999).
24. Pruitt, S.C., Mielnicki, L.M. & Stewart, C.C. Analysis of fluorescent protein expressing cells by flow cytometry. *Flow Cytometry Protocols* **263**, 239–258 (2004).
25. Ohnuki, M., Takahashi, K. & Yamanaka, S. *Generation and Characterization of Human Induced Pluripotent Stem Cells. Current Protocols in Stem Cell Biology* (John Wiley & Sons, Inc., 2007).
26. Strutt, B., Khalil, W. & Killinger, D. Growth and differentiation of human adipose stromal cells in culture. *Human Cell Culture Protocols* **2**, 41–56 (1996).
27. Xu, C. *et al.* Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotech.* **19**, 971–974 (2001).
28. Wagner, K. & Welch, D. Feeder-free adaptation, culture and passaging of human IPS cells using complete KnockOut Serum Replacement feeder-free medium. *J. Vis. Exp.* **15** (2010). Published online, doi:10.3791/2236.
29. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* **25**, 402–408 (2001).